[Frontiers in Bioscience 10, 689-698, January 1, 2005]

CRITICAL ROLE OF R-LOOPS IN PROCESSING REPLICATION BLOCKS

Manel Camps and Lawrence A. Loeb

The Joseph Gottstein Memorial Cancer Research Laboratory, Department of Pathology, University of Washington, Seattle, WA 98195-7705, USA

TABLE OF CONTENTS

1. ABSTRACT

Blocks in replication result from impediments to the advancing replication machinery and are lethal if not resolved. The replication fork must be reassembled for DNA synthesis to proceed. Fork assembly outside the chromosomal origin of replication (*oriC*) is mediated by recombination or *via* a helicase-dependent pathway. ColE1 plasmid origins of replication and *oriK* sites initiate primosome assembly by an RNA-DNA hybrid structure known as R-loop. We review evidence suggesting that R-loops are frequent during normal cell growth and that R-loops are critical for the maintenance of genome integrity. We propose that downstream of a replication block, RNA at R-loops is extended by DNA polymerase I, opening up the DNA duplex and leading to the recruitment of the replisome. This would allow replication to proceed while the original block is repaired or bypassed. Unlike recombination and helicase-dependent fork restoration, this mechanism would operate preferentially in transcribed areas of the genome, which are known to be particularly susceptible to DNA damage. Our model emphasizes the intimate relationship between transcription and repair, offers a unifying interpretation of phenotypes attributed to bacterial strains deficient in R-loop fork assembly, and calls for a renewed focus on R-loop formation and regulation.

2. INTRODUCTION

Blocks in DNA replication are caused by impediments such as lesions in the DNA, higher-order DNA structures, or the presence of proteins in the way of the advancing replication machinery. Replication arrest occurs during normal growth in culture and increases upon exposure to DNA-damaging agents (reviewed in (1)). Replication blocks lead to the inactivation of the replication fork and are lethal if left unresolved (2). For DNA synthesis to proceed, the replication machinery needs to be reassembled at the sites of replication arrest (reviewed in (3)).

Fork assembly at the chromosomal origin of replication (*oriC*) is mediated by a protein (DnaA) that opens up the DNA duplex. For fork assembly at sites other than *oriC*, melting of the DNA duplex is facilitated by hybridization of the template strand with an invading strand, which can be either DNA (generating a DNA-DNA hybrid called D-loop) or RNA (generating RNA-DNA hybrids known as R-loops). Upon opening of the duplex, primosome assembly is initiated by PriA. This protein recognizes forked structures such as D-loops and R-loops and loads the DnaB replicative helicase on the lagging strand (4,5). PriA also initiates primosome assembly in other specialized forms of DNA replication such as replication of certain plasmids and of phage but not during replicative chromosomal synthesis at OriC (reviewed in (6)).

D-loops result from recombination, and R-loops are associated with transcription (reviewed in (7)). R-loops form in the transcription bubble, probably by extension of existing 8-9 nt DNA-RNA hybrids within the RNA polymerase bubble. R-loops also occur behind the elongating transcription machinery, through invasion of the duplex by nascent RNA. This process is assisted by negative supercoiling and by Rec A.

Fork assembly at D-loops (known as recombination-dependent replication) is critical for double-strand break (DSB) repair (reviewed in (8)). R-loop-dependent fork assembly occurs at certain plasmid origins of replication and at *oriK* sites in the chromosome. Unlike D-loops, a possible role of

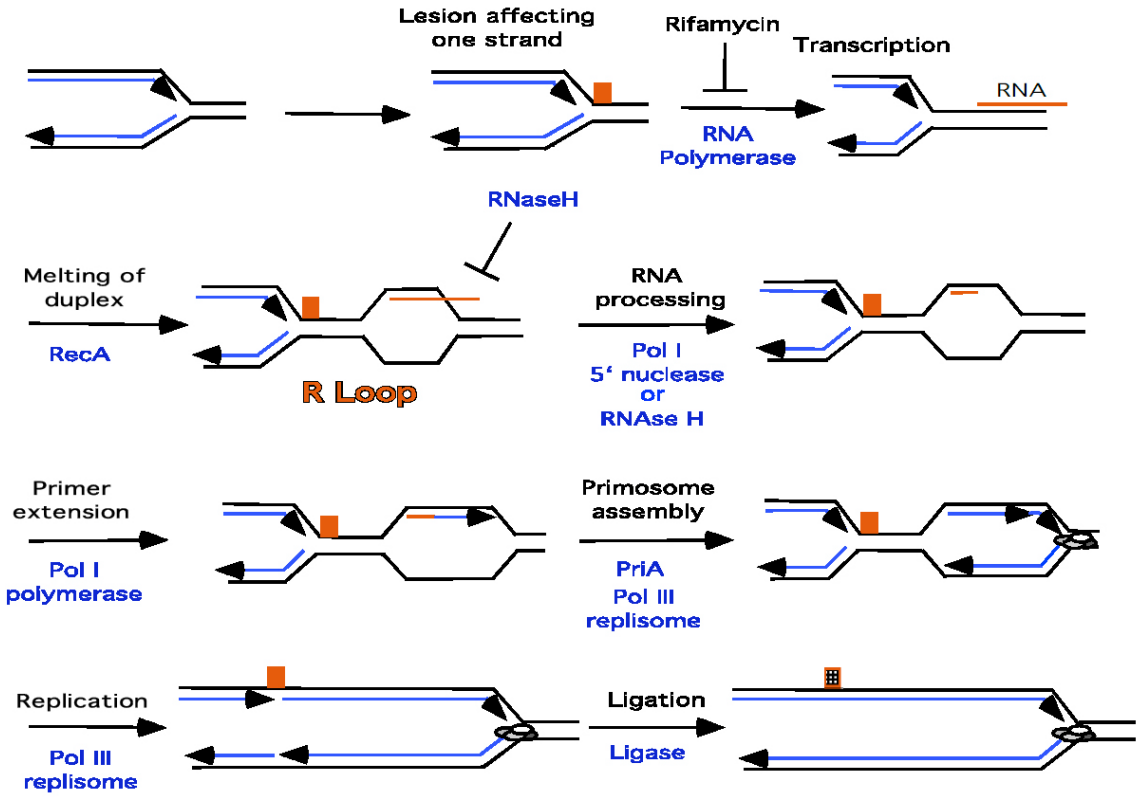

Figure 1. R-loop-dependent processing of inactivated forks. Individual steps are indicated above the arrows. The enzymatic functions mediating these steps are indicated underneath the arrows in blue font and discussed further in the text. A lesion that has either been repaired or bypassed is represented as hatched. Upon stalling of the leading strand, a DNA-RNA hybrid (R-loop) may form downstream of the lesion, originating either within the transcription bubble or through invasion of the DNA duplex by nascent RNA. Topological alterations in DNA in the context of stalled replication may favor R-loop formation by facilitating the opening of the DNA duplex. The 3'-hydroxyl of the transcript may serve as a primer for leading strand synthesis by Pol I. Processing by an exonuclease (such as RNase H or 5'→3' exonuclease of Pol I) likely facilitates priming but may not be essential. Synthesis of the leading strand would extend the bubble and allow loading of the Pol III replisome. The process of restarting replication would be completed when the original lesion is bypassed or repaired through ligation of the newly synthesized DNA to the DNA synthesized before the block occurred. Replication is allowed to continue while the original block is overcome. Only lesions affecting one strand of DNA are processed, as bypass or repair of the original lesion and ligation of the newly synthesized strands are required.

R-loops in processing replication blocks has not been established (reviewed in (9)).

In the present article, we propose that R-loops generated during transcription play a critical role in processing replication blocks during normal growth. Specifically, we propose that downstream of a replication block, the RNA present at an R-loop is extended by DNA polymerase I, opening up the DNA duplex and leading to PriA-mediated primosome assembly. We present an extensive analysis of the literature on *polA* strains that is consistent with this hypothesis. This novel mechanism for processing DNA blocks would be predicted to operate in actively transcribed areas of the genome. Given that these areas are more susceptible to DNA damage, R-loop-dependent fork assembly would provide an extra functional mechanism where it is more needed. Thus, our model emphasizes the intimate relationship between transcription and repair and offers a unifying interpretation of the phenomenology associated with strains deficient in R-loop fork assembly.

3. R-LOOP-DEPENDENT REPLICATION FORK ASSEMBLY RESTORES INACTIVATED REPLICATION FORKS

Replication fork assembly at R-loops may a play critical role in the cell beyond plasmid and *oriK* replication by restoring inactivated replication forks. A replication fork may assemble downstream of the relevant lesion at R-loops. Topological alterations in DNA in the context of stalled replication may favor R-loop formation by facilitating the opening of the DNA duplex.

By analogy with ColE1 plasmid replication, we propose that Pol I initiates primosome assembly by extending the 3'-hydroxyl of the transcript present in the R-loop (Figure 1). During ColE1 plasmid replication, a

transcript (RNAII) hybridizes to its complementary DNA sequence, generating an R-loop. The higher-order 3D structure of this DNA-RNA hybrid is recognized and processed by RNaseH I prior to elongation by Pol I. In the absence of RNaseH I, however, uncleaved DNA-RNA hybrids can be extended by Pol I, which attests to the ability of Pol I to extend unprocessed R-loops (10,11).

When the original lesion is bypassed or repaired, restoration of replication would be completed by joining of the newly synthesized DNA to the DNA synthesized before the block occurred (Figure 1). Thus, replication fork assembly at R-loops would allow replication to continue while the original block is processed. This mechanism would only affect lesions involving one strand of DNA, as bypass or repair of the original lesion and ligation of the two leader DNA strands (pre- and post-block) would be required.

The mechanism for processing replication blocks that we propose links replication restart to transcription. Transcription is known to increase mutagenesis (a phenomenon known as "transcription-associated mutation"), which presumably reflects an increased susceptibility of the displaced single strand to DNA damage (reviewed in (12)). Thus, R-loop-dependent fork processing would be facilitated in the areas that are most susceptible to replication blocks.

4. DNA POLYMERASE I

DNA polymerase I (Pol I) constitutes the majority of DNA polymerase activity in *E. coli*. The Pol I protein contains two domains: an N-terminal domain, a 5' nuclease formerly known as 5'→3' exonuclease or Exo II, and a C-terminal domain which combines a polymerase and a 3'→ 5' proofreading exonuclease. The N- and C-terminal domains are functionally independent, although their coordinated action is facilitated by being covalently linked. *In vitro*, on a nicked double-stranded DNA, the polymerase continuously regenerates the substrate for the 5'→3' exonuclease, resulting in a displacement of the nick along the duplex. Purified Pol I exhibits limited processivity *in vitro* (15-20 nts per DNA-binding event). This low processivity *in vitro* correlates *in vivo* with a role in gap processing during lagging strand synthesis and excision repair (reviewed in (13)).

4.1. Roles of Pol I beyond small gap processing

Beyond its role in processing small gaps, Pol I has the capacity to replicate long templates *in vivo*, at least in certain contexts. Examples include:

1. Pol I mediates DNA synthesis during long-patch base excision repair (>200 nucleotides) (14), and during nucleotide excision repair (>1500 nucleotides) (15).

2. Pol I synthesizes a long leading strand during initiation of ColE1 plasmid replication (up to 700bp) (16).

3. Synthesis of the leading strand exposes a primosome assembly site signal that is recognized by Pol III. Pol I appears be redundant with Pol III for completion of plasmid replication (reviewed in (17)).

4. The polymerase domain of Pol I is essential for replicative chromosomal synthesis in the absence of functional Pol III, again indicating a functional overlap with the more processive Pol III (18).

Replication by Pol I *in vivo* may be processive in nature. The well-defined point at which the switch from Pol I to Pol III occurs in ColE1-type plasmids, and the extent of DNA synthesis that must be involved in chromosomal replication in Pol III-deficient strains are suggestive of processive synthesis. Processivity by Pol I would involve the recruitment of processivity factors. There is indeed evidence that Pol I binds the β-clamp, and that formation of this complex dramatically enhances the processivity of Pol I DNA synthesis in *vitro* (19). However, replication of extensive segments of DNA *in vivo* by a distributive mechanism cannot be excluded given the relative abundance of Pol I in *E. coli* (400 molecules/cell (13)).

4.2. PolA and *priA* strains exhibit similar phenotypes

A large number of studies on *polA* strains has been reported. A variety of polA alleles have been used, making a comparative analysis of these reports difficult. In the present manuscript, we made an effort to identify specific alleles and to justify generalizations when we group several of them together. The most frequently used mutants are presented in Table 1. The *PolA12* mutation exhibits a temperature-sensitive defect in polymerase activity (20) that has been invaluable to study epistatic interactions of Pol I with other genes (Table 2). Strains with a significant deficiency in Pol I polymerase activity show the following phenotypic manifestations, summarized in Table 3:

1. No growth in rich medium on solid agar.

2. Poor growth and low viability in liquid rich medium.

3. Constitutive SOS expression, resulting in filamentous growth and in increased mutagenesis. Filamentous growth occurs early in microcolony development and is suppressed at high cell density.

4. Failure to maintain ColE1-type plasmids (ColE1, ColE2, pBR322, RSF1030) but ability to replicate DnaA-type plasmids (F, pSC101, R6K).

5. Increased sensitivity to UV and MMS.

6. Increased sensitivity to γ-irradiation.

7. Defects in F plasmid-mediated conjugation and in homologous recombination.

PriA strains exhibit a strikingly similar behavior, although the recombination and repair phenotypes are more prominent in *priA* cells (Table 3). The *polA12* mutation has been reported to be synthetic lethal with *priA,* and this effect has been attributed the synergy between defective

Table 1. Most frequently reported *Pol A* mutations

Allele	Mutation	Amino Acid substitution	Location	Ref.	5'→3' exo activity	Polymerase activity	Ref.
polA1	G(1025) →A	W(342) →Am	N-terminal end of polymerase	62	Normal	Low levels	20
resA1	C(892) →T	Q(286) →Am	C-terminal end of 5' nuclease	62	Normal	Low levels	63
polA12	G(1631) →A	G(544)→D	Motif 1 in polymerase domain	[1]	Normal	Temperature-sensitive [3]	20
polA2099::Tn	Tn insertion, duplicating base pairs 2064 through 2612	Deletion of last 58 amino acids	C-terminus, deleting motif C	64	Normal [2]	Low	64
polA107	A(230) →G	Y(77)→C	5' nuclease domain	62	Low levels	normal	65

[1] J. Naukkarinen and M. Camps, personal communication. [2] Predicted based on the nature and location of the mutation. [3] The purified protein is misfolded and defective in nick translation *in vitro* (66).

Table 2. Mutations that enhance the phenotype of *polA* strains

Increased Replication Block		Ref.
rnhA [1]	*polA12*	24
recA441 [2]	*polA12* [3]	44
recA730	*polA12* [3]	
recA718 [4]	*polA1, polA12*	53
Impaired Fork Rescue		**Ref.**
priA	*polA12*	67
recG	*polA1, polA12*	25
recA [5]	*polA1, polA12*	68,69 [6]
recBC	*polA12*	70 [7]
ruvAB	*polA12*	30 [8]

[1] RNaseHI encoded by *rnhA* suppresses unscheduled R-loop formation (for a review see (71)). [2] *recA441* and *recA730* are mutants which express the SOS response constitutively (72). [3] The polymerase activity is the one which is essential for survival in the presence of the *recA441* and *recA730* mutations (44). [4] *recA718* is primed for SOS activation but retains proficiency in recombination and in all RecA proteolytic activities (43).[5] Only the recombinase function is required (39). [6] Associated with DNA degradation (69,70). [7] Not associated with DNA degradation (70). [8] Cited as unpublished data.

Table 3. *polA* and *priA* strains exhibit similar phenotypes

Phenotype	*pol A*	Specific alleles	Ref.	*priA* [1]	Ref.
Sensitivity to rich medium on solid agar	+++	*ΔpolA*	40,73	+++	41
Reduced viability in liquid medium	+++	*polA1*	32	+++	41,67
Constitutive SOS induction	+++	*polA2099::mini-Tn10*	64	+++	74
Filamentous growth	+++	*polA2099::mini-Tn10*	64	+++	67,74
Reduced maintenance of non DnaA-dependent plasmids	+++	*polA1, resA1*	49,75	+++	41,67,76
Sensitivity to UV	++	*polA1, ΔpolA, polA546* [2]	32,33	+++	67,74
Sensitivity to γ-irradiation	++	*polA1*	77,78	+++	67,79
Poor homologous recombination	+ [3]	*polA1, polA107*	80,81	+++	74,79

[1] These are null alleles by insertion or replacement with a *kan* gene (67, 76). Since *priA* deletion causes severe broth sensitivity, and the original stains were isolated in LB broth, they most likely contain suppressor mutations (41). [2] Allele with a temperature-sensitive 5' →3' exonuclease activity. [3] One *polA* strain has been reported to be hyperrecombinogenic in the Konrad assay (82). This assay detects restoration of the *lacZ* gene in cells encoding two inversely oriented *lacZ* sequences with non-overlapping deletions. In this case, hyperrecombination may be attributed to increased nick formation and replication fork collapse in the absence of Pol I, which is likely to lead to erroneous pairing when replication resumes (83).

DSB repair (*priA* strains) and increased DSB formation associated with defective gap filling (*polA* strains)(8). The striking parallel in phenotypic profiles between *polA* and *priA* strains, and the fact they are distinct from phenotypes of other players in DNA repair suggests critical roles of Pol I which are related to those of PriA.

4.3. Pol I is critical for R-loop extension

There are two lines of evidence implicating Pol I in replication at R-loops. The most direct evidence is that deletion of polA selectively inhibits rifamycin-sensitive stable DNA replication. The second line of evidence comes from strains deficient in a suppressor of R-loop formation (RNase H I).

Stable DNA replication (SDR) represents replication initiated at sites other than OriC. SDR is chloramphenicol-resistant and typically requires SOS induction. Initiation at R-loops is associated with transcription and is therefore susceptible to inhibition by rifamycin, whereas initiation at D-loops is rifamycin-resistant. In one study, which used thymidine starvation to induce SOS, Δ*polA* cells showed a clear inhibition of rifamycin-sensitive SDR (21). This contradicts an earlier study showing no effect on rifamycin-sensitive SDR, but those results need to be regarded with caution because SOS was induced by UV irradiation, which has notoriously pleoitropic effects (22). Rifamycin-resistant SDR, on the other hand was not inhibited in either study (21,22). Pol I deficiency has only a moderate effect on UV and γ-irradiation sensitivity relative to PriA deficiency (Table 3), which is remarkable considering that *polA* cells show increased nick and gap formation. Thus, *polA* strains defective in polymerase activity appear to be competent for D-loop but not R-loop formation. This is suggested by the moderate UV and γ-irradiation sensitivity (considering that recombination-dependent DNA replication is critical for DSB repair), by the fact that these *polA* strains show minimal recombination phenotypes (Table 3), and the little effect of a *polA* deletion on rifamycin-resistant SDR. Overall, these observations point to a role of Pol I in fork assembly that is specific for R-loop–dependent initiation.

The second line of evidence derives from observations that are consistent with an increase in unprocessed R-loops in strains deficient in Pol I polymerase activity. Unprocessed R-loops are deleterious, as they represent blocks in DNA replication (23,24). RNase H I (the product of the *rnhA* gene) is a strong suppressor of R-loop formation, hydrolyzing DNA-RNA hybrids. *RnhA* strains therefore show enhanced R-loop formation. The growth phenotype of *rnhA* cells is very similar to that of *polA* cells, including sensitivity to rich medium, constitutive expression of the SOS response, and filamentous growth (24). This observation agrees with the hypothesis that *polA* strains suffer from excessive R-loop formation. Along the same lines, *rnhA polA 12* cells are temperature-sensitive for growth (25).

5.REDUNDANCIES OF THE R-LOOP-DEPENDENT PATHWAY OF FORK ASSEMBLY

Blocks in DNA replication may result from damage to one or to both DNA strands. In the case of damage to a single strand, lesions affecting the leading strand template are more likely to block the advance of the replication machinery, as the synthesis of the lagging strand is discontinuous by nature and hence more tolerant of stalling. Damage affecting both strands, such as double strand breaks (DSB) or crosslinks, invariably blocks replication.

Regression of a blocked fork allows the complementary nascent strands to anneal and to form a Holliday junction (HJ) (reviewed in (26)). This HJ can be resolved by at least two pathways, both involving PriA-mediated fork assembly: "recombination-dependent DNA replication", and "fork dereversal". The recombination-dependent DNA replication pathway resolves the HJ through a resolvase (RuvABC) followed by a recombinase-mediated strand exchange (RecA and RecBCD). This leads to the formation of D-loops. PriA mediates fork assembly at these recombination intermediates though its primosome activity. In the "fork dereversal" pathway, fork assembly at the HJ is achieved by direct primosome assembly through the coordinated action of the RecG and PriA helicases (27). Recombination-dependent replication has the capacity to resolve blocks involving one or both strands of DNA, whereas the more direct mechanism of fork dereversal is limited to blocks involving a single strand of DNA. *priA* strains show a severer phenotype than *recA*, *recB*, *ruvABC*, or *recG* strains, as expected given the central role of PriA in both the "recombination-dependent DNA replication" and "fork dereversal" pathways.

Thus, fork assembly at R-loops would be redundant with both the recombination-dependent DNA replication and with fork dereversal pathways (diagrammed in Figure 2). This functional overlap probably masked the delineation of this mechanism in the past.

Recombination-dependent DNA replication resolves blocks involving one strand as well as blocks involving both DNA strands such as double-strand breaks and crosslinks. Sites of DNA damage to one strand, if not removed, are prone to cause DSBs during replication, transcription, or recombination (reviewed in (28)). Thus, recombination-dependent DNA replication may function as a general backup mechanism, processing any persistent block when direct reversal is delayed or impossible (Figure 2). *PolA* is deleterious in combination with mutations in the genes that specifically mediate recombination-dependent DNA replication: *recB*, and *ruvAB* (Table 3). These epistatic interactions are consistent with the existence of an alternative mechanism of fork restoration that is Pol I-dependent (the R-loop-dependent pathway), although increased nick and gap formation associated with the *polA* mutation likely contributes to the synthetic lethality as well.

"Fork dereversal", i.e. direct primosome assembly at the site of fork reversal was discovered in cells deficient in recombination because in these strains (*recB* or *ruvA*) the *recG* mutation increases UV sensitivity to levels comparable to those of *priA* cells (27). The helicase activities of PriA itself and of RecG are required in this case because DnaB needs to be loaded on the strand opposite to the displaced single strand (27). The helicase activity of RecG also catalyzes reverse branch migration and the interconversion of replication forks and HJ structures during recombination (29) and resolves R-loops (25,30). The mild growth phenotype of *recG* cells (30) suggests that this pathway is of limited significance during normal growth. The synthetic lethality of *recG* and *polA* mutations (25) is likely due delayed processing of R-loops because of deficiencies in RecG-mediated R-loop resolution and in PolA-dependent fork assembly.

The relative importance of each of these pathways to process stalled forks needs to be established.

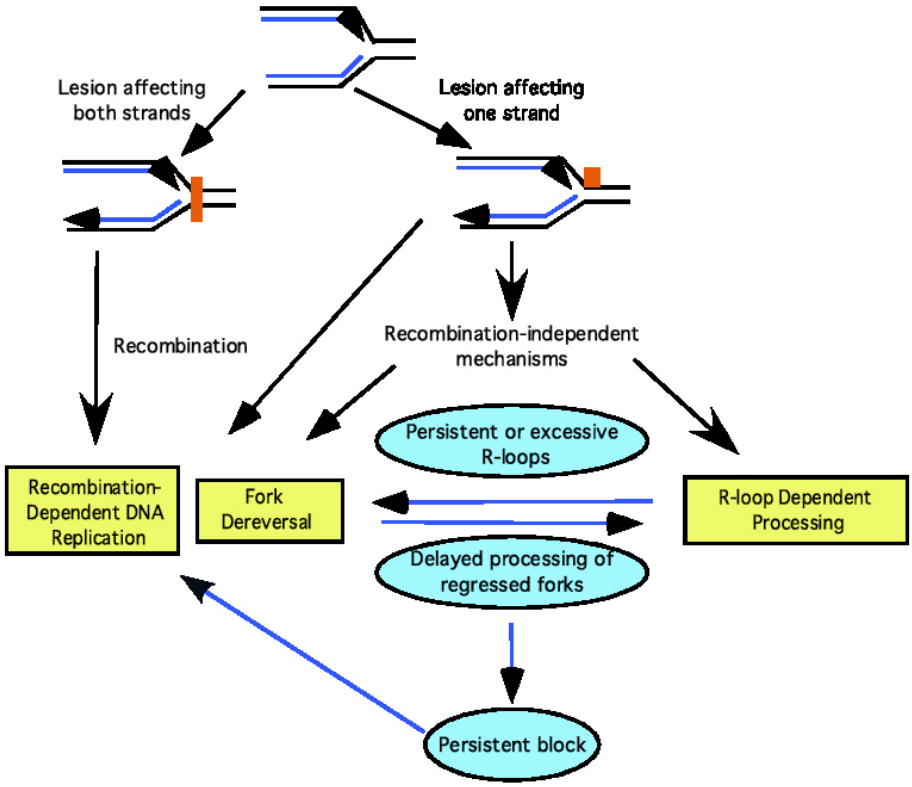

Figure 2. Functional overlap between the three putative pathways to process stalled forks. The three putative pathways for processing replication forks mentioned in the text are boxed in yellow and compensating interactions are presented in blue ovals. The fork dereversal pathway would be expected to overlap with R-loop-dependent processing. This pathway would become more important in situations of increased R-loop formation (*rnhA*, certain RNA pol mutations, SOS expression, etc…) or of delayed processing (*polA*). Conversely, impairment of the fork dereversal pathway or of recombination-dependent replication would increase the cell's dependence on fork assembly at R-loops. The similar growth phenotype of *polA* and *priA* strains contrasts with the mild growth phenotype of *recG*, *recB*, and *ruvAB* cells, suggesting that fork assembly at R-loops is of likely functional relevance during growth in culture (Figure 1). Sites of damaged DNA are fragile and prone to break during replication, transcription or recombination. Thus, recombination-dependent replication may function as a general backup mechanism, processing any persistent block regardless of when direct reversal is delayed or impossible.

The fact that *polA* and *priA* strains show strikingly similar growth phenotypes in spite of evidence that *polA* strains remain competent for recombination-dependent DNA replication suggests that fork assembly at R-loops may be of considerable functional significance during rapid growth in culture.

6. PREDICTIONS

The novel mechanism to process stalled forks presented here emphasizes the intimate relationship between transcription and repair and offers a unifying interpretation of phenotypes attributed to bacterial strains deficient in R-loop fork assembly. Specifically, our model makes the following two predictions:

6.1. Conditions that promote replication blocks affecting one strand of DNA should sensitize *polA* cells and enhance rifamycin-sensitive SDR

Examples of such conditions include DNA damage by exogenous agents, growth in rich medium, constitutive SOS expression, and modifications of the transcriptional machinery.

PolA strains deficient in polymerase activity are hypersensitive to UV irradiation and to MMS treatment (31-34). Given that these strains are defective in short gap repair, this observation cannot be unambiguously interpreted. Significantly, though, replication associated with repair of UV lesions ("induced replisome reactivation" or IRR) is sensitive to rifamycin (35) and depends on overproduction of RecA (36,37), suggesting R-loop involvement. Moreover, overproduction of RNaseH I sensitizes cells to UV irradiation and interferes with IRR (38), which is strong evidence that R-loops are involved in restarting replication after UV damage.

Initiation of replication occurs more frequently in rich media, effectively increasing the potential for blockage of the replication machinery (39). *Pol A* cells (like *rnhA*, and *priA* cells) are sensitive to growth in rich media (24,40,41). Upon entry into stationary phase, cells growing in rich media show SDR activity that is partially rifamycin-

sensitive and dependent on RecA but not RecB (42), pointing to R-loop involvement.

Activation of the SOS response through the *recA441*, *recA730*, and *recA718* mutations is lethal in *polA1* and *polA12* strains (Table 3). The *RecA718* allele is competent in recombination and in RecA-mediated proteolytic activities (43), confirming that lethality is caused by expression of SOS *per se* rather than due to defects in RecA-mediated repair. The detrimental effect of constitutive SOS expression in these *polA* strains has defied explanation, as it has no significant effect on the processing of Okazaki fragments (44). In light of our model, we propose that the synthetic lethality is caused by the compounded effects of increased replication arrest associated with SOS expression and defective R-loop-mediated processing of these blocks due to defective *polA* polymerase function.

Mutations in *rpoB* appear to modulate R-loop formation, likely by influencing the size of DNA-RNA hybrids (24,45). A subset of mutations in RNA polymerase (*ropB**) protect *ruvAC* cells from UV damage (46). These mutations increase tolerance of the RNA polymerase for stalling at sites of DNA lesions (46,47) and are also likely to have an effect on R-loop formation because they disrupt interactions essential for stable DNA binding (47).

6.2. The 5' Nuclease domain should not be essential for R loop-dependent processing of replication forks

Reconstituted initiation of ColE1 plasmid replication *in vitro* requires both the polymerase and the 5' nuclease domains of Pol I, suggesting that nick translation is limiting for plasmid initiation *in vitro* (48). The 5' nuclease domain, however, appears to be dispensable for ColE1 plasmid replication *in vivo* (49-52) (not in all cases (21)), indicating that primer extension is often the limiting activity *in vivo*. By analogy to ColE1 plasmid replication, fork assembly at R-loops would be expected to be mostly dependent on the polymerase domain of Pol I. In agreement this prediction, the 5' nuclease activity is not required for synthetic lethality of *polA12* in combination with *recA441* or *recA730* (44). Further, *polA12 rec718* cells have been complemented by a variety of polymerases lacking 5' $\rightarrow$3' exonuclease activity , including overexpression of the *E. coli* DnaE (α subunit of Pol III), human Pol-β, HIV reverse transcriptase, and *T. aquaticus* DNA (*Taq*) polymerase (53-56). In all cases except *Taq,* complementation correlates with restoring ability of *polA12 recA718* cells to support ColE1 plasmid replication at the restrictive temperature, which further supports the proposition that extension of an RNA primer is limiting in *polA12 rec718* cells (52,53,56).

Interestingly, the 5' nuclease domain on its own complements the sensitivity to rich medium of a *polAΔ* strain (40) and the synthetic lethality of *polA12 recA718* cells (53), although it has not been reported to complement initiation of ColE1 plasmid replication. This suggests that the 5' nuclease domain of Pol I likely plays a role in restoring stalled replication by a different mechanism. Indeed, there is evidence that the 5' nuclease domain of Pol I is involved in recombination-dependent replication, possibly promoting strand exchange (21,33,57).

7. PERSPECTIVES

We review evidence of the critical role of R-loop fork assembly during normal cell growth. We propose that R-loops generated during transcription can be extended by DNA polymerase I to initiate primosome assembly by a mechanism reminiscent of initiation of ColE1 plasmid replication. If fork assembly occurs in proximity downstream of a replication block, it could allow replication to proceed while the original replication block is repaired or bypassed. Our model has implications for understanding of mechanisms of DNA repair in multicellular organisms, as homologues of the polymerase domain of Pol I have been found in Drosophila (*Mus308)* (58) and in humans [POL N (59) and POL Q (60)].

The mechanism of R-loop-dependent processing of stalled forks that we propose awaits further confirmation. Confirming the epistatic interactions between *polA*, *rpoB* mutations that increase R-loop formation, *RecA* mutations leading to constitutive SOS expression, and mutations in the other players involved in restoration of fork assembly (*recG* and *ruvAB)* predicted by our model will be of great interest. R-loop formation downstream, adjacent to replication blocks also needs to be established. Reconstitution of R-loop formation with a nascent transcript *in vitro* would represent an important first step in this direction. The RecA-catalyzed assimilation of complementary RNA into a homologous region of a duplex has recently been achieved (61). If confirmed, our model calls for further studies on R-loop regulation and on the relative contribution of each pathway of fork restoration .

Unlike recombination and helicase-dependent fork restoration, R loop-dependent processing of stalled forks would operate preferentially in transcribed areas of the genome, which are especially susceptible to DNA damage. This highlights the intimate relationship between transcription and repair. Our model offers a unifying interpretation of phenotypes attributed to bacterial strains deficient in R-loop fork assembly, and calls for a renewed focus on R-loop formation and regulation.

9. ACKNOWLEDGEMENTS

This work was supported by National Institute of Health Grants CA78885 and CA80993 (LA Loeb), and by the ES0732-25 training grant of Environmental Pathology/Toxicology (MC). We are grateful to Dr. Joann Sweasy, Dr. Raymond Monnat, Dr. Roel Schaaper, and Dr. Ann Blank for critical reading on the manuscript. Manel Camps wants to dedicate this work to his wife Teresa McCaffrey and to his friend Martí Duran.

10. REFERENCES

1. M. M. Cox *et al.*:The importance of repairing stalled replication forks. *Nature* 404, 37-41 (2000)
2. Horiuchi T. & Y. Fujimura: Recombinational rescue of the stalled DNA replication fork: a model based on analysis of an Escherichia coli strain with a chromosome region difficult to replicate. *J Bacteriol* 177, 783-91 (1995)

3. K. J. Marians: PriA-directed replication fork restart in Escherichia coli. *Trends Biochem Sci* 25, 185-9 (2000)
4. Liu J. & K. J. Marians: PriA-directed assembly of a primosome on D loop DNA. *J Biol Chem* 274, 25033-41 (1999)
5 McGlynn P., Al-Deib A. A., Liu J., Marians K. J. & R. G. Lloyd: The DNA replication protein PriA and the recombination protein RecG bind D-loops. *J Mol Biol* 270, 212-21 (1997)
6. Masai H. & K. I. Arai: DnaA- and PriA-dependent primosomes: Two distinct replication complexes for replication of Escherichia coli chromosome. *Front Biosci* 1, d48-58 (1996)
7. M. Drolet, *et al*.: The problem of hypernegative supercoiling and R-loop formation in transcription. *Front Biosci* 8, d210-21 (2003)
8. T. Kogoma: Recombination by replication. *Cell* 85, 625-7 (1996)
9. T. Kogoma: Stable DNA replication: interplay between DNA replication, homologous recombination, and transcription. *Microbiol Mol Biol Rev* 61, 212-38 (1997)
10. Naito S. & H. Uchida: RNase H and replication of ColE1 DNA in *Escherichia coli*. *J Bacteriol* 166, 143-7 (1986)
11. Dasgupta S., Masukata H. & J. Tomizawa: Multiple mechanisms for initiation of ColE1 DNA replication: DNA synthesis in the presence and absence of ribonuclease H. *Cell* 51, 1113-22 (1987)
12. A. Aguilera: The connection between transcription and genomic instability. *Embo J* 21, 195-201 (2002)
13. A Kornberg & T Baker: DNA Polymerase I of *E. coli* In: DNA Replication*: W.H. Freeman and Company*, New York, 113-164 (1992)
14. Sung J. S. & D. W. Mosbaugh: *Escherichia coli* uracil- and ethenocytosine-initiated base excision DNA repair: rate-limiting step and patch size distribution. *Biochemistry* 42, 4613-25 (2003)
15. P. K. Cooper: Characterization of long patch excision repair of DNA in ultraviolet-irradiated *Escherichia coli*: an inducible function under rec-lex control. *Mol Gen Genet* 185, 189-97 (1982)
16. Camps M., Naukkarinen J., Johnson B. P. & L. A. Loeb: Targeted gene evolution in *Escherichia coli* using a highly error-prone DNA polymerase I. *Proc Natl Acad Sci U S A* 100, 9727-32 (2003)
17. Camps M. & L. A. Loeb: When Pol I goes into high gear; processive DNA synthesis by Pol I in the cell. *Cell Cycle* 3, 1-3 (2004)
18. Bryan S. K. & R. E. Moses: Sufficiency of the Klenow fragment for survival of polC(Ts) pcbA1 *Escherichia coli* at 43 degrees C. *J Bacteriol* 170, 456-8 (1988)
19. Lopez de Saro F. J. & M. O'Donnell: Interaction of the beta sliding clamp with MutS, ligase, and DNA polymerase I. *Proc Natl Acad Sci U S A* 98, 8376-80 (2001)
20. Lehman I. R. & J. R. Chien: Persistence of deoxyribonucleic acid polymerase I and its 5'--3' exonuclease activity in PolA mutants of *Escherichia coli* K12. *J Biol Chem* 248, 7717-23 (1973)
21. Kogoma T. & R. R. Maldonado: DNA polymerase I in constitutive stable DNA replication in *Escherichia coli*. *J Bacteriol* 179, 2109-15 (1997)
22. Ruscitti T. & S. Linn: DNA polymerase I modulates inducible stable DNA replication in *Escherichia coli*. *J Bacteriol* 174, 6311-3 (1992)
23. Itaya M. & R. J. Crouch: A combination of RNase H (*rnh*) and *recBCD* or *sbcB* mutations in *Escherichia coli* K12 adversely affects growth. *Mol Gen Genet* 227, 424-32 (1991)
24. Kogoma T., Hong X., Cadwell G. W., Barnard K. G. & T. Asai: Requirement of homologous recombination functions for viability of the *Escherichia coli* cell that lacks RNase HI and exonuclease V activities. *Biochimie* 75, 89-99 (1993)
25. Hong X., Cadwell G. W. & T. Kogoma: *Escherichia coli* RecG and RecA proteins in R-loop formation. *Embo J* 14, 2385-92 (1995)
26. B. Michel, et al.: Rescue of arrested replication forks by homologous recombination. *Proc Natl Acad Sci U S A* 98, 8181-8 (2001)
27. Gregg A. V., McGlynn P., Jaktaji R. P. & R. G. Lloyd: Direct rescue of stalled DNA replication forks via the combined action of PriA and RecG helicase activities. *Mol Cell* 9, 241-51 (2002)
28. B. Michel: Replication fork arrest and DNA recombination. *Trends Biochem Sci* 25, 173-8 (2000)
29. McGlynn P. & R. G. Lloyd: Rescue of stalled replication forks by RecG: simultaneous translocation on the leading and lagging strand templates supports an active DNA unwinding model of fork reversal and Holliday junction formation. *Proc Natl Acad Sci U S A* 98, 8227-34 (2001)
30. Ishioka K., Iwasaki H. & H. Shinagawa: Roles of the recG gene product of Escherichia coli in recombination repair: effects of the delta *recG* mutation on cell division and chromosome partition. *Genes Genet Syst* 72, 91-9 (1997)
31. Gross J. & M. Gross: Genetic analysis of an *E. coli* strain with a mutation affecting DNA polymerase. *Nature* 224, 1166-8 (1969)
32. Monk M., Peacey M. & J. D. Gross: Repair of damage induced by ultraviolet light in DNA polymerase- defective *Escherichia coli* cells. *J Mol Biol* 58, 623-30 (1971)
33. Sharma R. C. & K. C. Smith: Role of DNA polymerase I in postreplication repair: a reexamination with *Escherichia coli* delta *polA*. *J Bacteriol* 169, 4559-64 (1987)
34. H. Bates *et al*.: Spontaneous and UV-induced mutations in *Escherichia coli* K-12 strains with altered or absent DNA polymerase I. *J Bacteriol* 171, 2480-4 (1989)
35. Khidhir M. A., Casaregola S. & I. B. Holland: Mechanism of transient inhibition of DNA synthesis in ultraviolet- irradiated *E. coli*: inhibition is independent of recA whilst recovery requires RecA protein itself and an additional, inducible SOS function. *Mol Gen Genet* 199, 133-40 (1985)
36. Casaregola S., Khidhir M. & I. B. Holland: Effects of modulation of RNase H production on the recovery of DNA synthesis following UV-irradiation in *Escherichia coli*. *Mol Gen Genet* 209, 494-8 (1987)

37. Witkin E. M., Roegner-Maniscalco V., Sweasy J. B. & J. O. McCall: Recovery from ultraviolet light-induced inhibition of DNA synthesis requires umuDC gene products in *recA718* mutant strains but not in *recA+* strains of *Escherichia coli*. *Proc Natl Acad Sci U S A* 84, 6805-9 (1987)
38. Bockrath R., Wolff L., Farr A. & R. J. Crouch: Amplified RNase H activity in *Escherichia coli* B/r increases sensitivity to ultraviolet radiation. *Genetics* 115, 33-40 (1987)
39. Cao Y. & T. Kogoma: The mechanism of *recA polA* lethality: suppression by RecA-independent recombination repair activated by the lexA(Def) mutation in *Escherichia coli*. *Genetics* 139, 1483-94 (1995)
40. Joyce C. M. & N. D. Grindley: Method for determining whether a gene of *Escherichia coli* is essential: application to the *polA* gene. *J Bacteriol* 158, 636-43 (1984)
41. Masai H., Asai T., Kubota Y., Arai K. & T. Kogoma: *Escherichia coli* PriA protein is essential for inducible and constitutive stable DNA replication *Embo J* 13, 5338-45 (1994)
42. Hong X., Cadwell G. W. & T. Kogoma: Activation of stable DNA replication in rapidly growing *Escherichia coli* at the time of entry to stationary phase. *Mol Microbiol* 21, 953-61 (1996)
43. McCall J. O., Witkin E. M., Kogoma T. & V. Roegner-Maniscalco: Constitutive expression of the SOS response in *recA718* mutants of *Escherichia coli* requires amplification of RecA718 protein. *J Bacteriol* 169, 728-34 (1987)
44. Fijalkowska I., Jonczyk P. & Z. Ciesla: Conditional lethality of the *recA441* and *recA730* mutants of *Escherichia coli* deficient in DNA polymerase I. *Mutat Res* 217, 117-22 (1989)
45. T. Kogoma: *Escherichia coli* RNA polymerase mutants that enhance or diminish the SOS response constitutively expressed in the absence of RNase HI activity. *J Bacteriol* 176, 1521-3 (1994)
46. McGlynn P. & R. G. Lloyd: Modulation of RNA polymerase by (p)ppGpp reveals a RecG-dependent mechanism for replication fork progression. *Cell* 101, 35-45 (2000)
47. Trautinger B. W. & R. G. Lloyd: Modulation of DNA repair by mutations flanking the DNA channel through RNA polymerase. *Embo J* 21, 6944-53 (2002)
48. Itoh T. & J. Tomizawa: Initiation of replication of plasmid ColE1 DNA by RNA polymerase, ribonuclease H, and DNA polymerase I. *Cold Spring Harb Symp Quant Biol* 43, 409-17 (1979)
49. Tacon W. & D. Sherratt: ColE plasmid replication in DNA polymerase I-deficient strains of *Escherichia coli*. *Mol Gen Genet* 147, 331-5 (1976)
50. Grindley N. D. & W. S. Kelley: Effects of different alleles of the E. coli K12 pol A gene on the replication of non-transferring plasmids. *Mol Gen Genet* 143, 311-8 (1976)
51. Veltkamp E. & H. J. Nijkamp: The role of DNA polymerase I, II and 3 in the replication of the bacteriocinogenic factor Clo DF 13. *Mol Gen Genet* 125, 329-40 (1973)
52. Sweasy J. B., Chen M. & L. A. Loeb: DNA polymerase beta can substitute for DNA polymerase I in the initiation of plasmid DNA replication. *J Bacteriol* 177, 2923-5 (1995)
53. Witkin E. M. & V. Roegner-Maniscalco: Overproduction of DnaE protein (alpha subunit of DNA polymerase III) restores viability in a conditionally inviable *Escherichia coli* strain deficient in DNA polymerase I. *J Bacteriol* 174, 4166-8 (1992)
54. Sweasy J. B. & L. A. Loeb: Mammalian DNA polymerase beta can substitute for DNA polymerase I during DNA replication in *Escherichia coli*. *J Biol Chem* 267, 1407-10 (1992)
55. Suzuki M., Baskin D., Hood L. & L. A. Loeb: Random mutagenesis of *Thermus aquaticus* DNA polymerase I: concordance of immutable sites in vivo with the crystal structure. *Proc Natl Acad Sci U S A* 93, 9670-5 (1996)
56. Kim B. & L. A. Loeb: Human immunodeficiency virus reverse transcriptase substitutes for DNA polymerase I in *Escherichia coli*. *Proc Natl Acad Sci U S A* 92, 684-8 (1995)
57. Zhang W. & D. H. Evans: DNA strand transfer catalyzed by the 5'-3' exonuclease domain of *Escherichia coli* DNA polymerase I. *Nucleic Acids Res* **2**3, 4620-7 (1995)
58. P. V. Harris *et al*.: Molecular cloning of Drosophila *mus308*, a gene involved in DNA cross-link repair with homology to prokaryotic DNA polymerase I genes. *Mol Cell Biol* 16, 5764-71 (1996)
59. Sharief F. S., Vojta P. J., Ropp P. A. & W. C. Copeland: Cloning and chromosomal mapping of the human DNA polymerase theta (POLQ), the eighth human DNA polymerase. *Genomics* 59, 90-6 (1999)
60. Marini F., Kim N., Schuffert A. & R. D. Wood: POLN, a nuclear PolA family DNA polymerase homologous to the DNA cross-link sensitivity protein *Mus308*. *J Biol Chem* 278, 32014-9 (2003)
61. Kasahara M., Clikeman J. A., Bates D. B. & T. Kogoma: RecA protein-dependent R-loop formation in vitro. *Genes Dev* 14, 360-5 (2000)
62. Joyce C. M., Fujii D. M., Laks H. S., Hughes C. M. & N. D. Grindley: Genetic mapping and DNA sequence analysis of mutations in the *polA* gene of *Escherichia coli*. *J Mol Biol* 186, 283-93 (1985)
63. Glickman B. W., van Sluis C. A., van der Maas G. & A. Rorsch: Comparison of the *resA1* and *polA1* mutations in isogenic strains of *Escherichia coli* K-12. *J Bacteriol* 114, 951-5 (1973)
64. J. A. Shapiro: Differential action and differential expression of DNA polymerase I during *Escherichia coli* colony development. *J Bacteriol* 174, 7262-72 (1992)
65. H. L. Heijneker *et al*.: A mutant of *Escherichia coli* K12 deficient in the 5'-3' exonucleolytic activity of DNA polymerase I. II. Purification and properties of the mutant enzyme. *Mol Gen Genet* 124, 83-96 (1973)
66. Uyemura D. & I. R. Lehman: Biochemical characterization of mutant forms of DNA polymerase I from *Escherichia coli*. I. The *polA12* mutation. *J Biol Chem* 251, 4078-84 (1976)
67. Lee E. H. & A. Kornberg: Replication deficiencies in *priA* mutants of *Escherichia coli* lacking the primosomal replication n' protein. *Proc Natl Acad Sci U S A* 88, 3029-32 (1991)

68. Gross J. D., Grunstein J. & E. M. Witkin: Inviability of *recA*- derivatives of the DNA polymerase mutant of De Lucia and Cairns. *J Mol Biol* 58, 631-4 (1971)
69. Lloyd R. G., Low B., Godson G. N. & E. A. Birge: Isolation and characterization of an *Escherichia coli* K-12 mutant with a temperature-sensitive *recA*- phenotype. *J Bacteriol* 120, 407-15 (1974)
70. Monk M. & J. Kinross: Conditional lethality of *recA* and *recB* derivatives of a strain of *Escherichia coli* K-12 with a temperature-sensitive deoxyribonucleic acid polymerase I. *J Bacteriol* 109, 971-8 (1972)
71. Asai T. & T. Kogoma: D-loops and R-loops: alternative mechanisms for the initiation of chromosome replication in *Escherichia coli*. *J Bacteriol* 176, 1807-12 (1994)
72. Witkin E. M. & T. Kogoma: Involvement of the activated form of RecA protein in SOS mutagenesis and stable DNA replication in *Escherichia coli*. *Proc Natl Acad Sci U S A* 81, 7539-43 (1984)
73. Moolenaar G. F., Moorman C. & N. Goosen: Role of the *Escherichia coli* nucleotide excision repair proteins in DNA replication. *J Bacteriol* 182, 5706-14 (2000)
74. Sandler S. J., Samra H. S. & A. J. Clark: Differential suppression of *priA2::kan* phenotypes in *Escherichia coli* K- 12 by mutations in *priA, lexA,* and *dnaC*. *Genetics* 143, 5-13 (1996)
75. Kingsbury D. T. & D. R. Helinski: Temperature-sensitive mutants for the replication of plasmids in *Escherichia coli*: requirement for deoxyribonucleic acid polymerase I in the replication of the plasmid ColE 1. *J Bacteriol* 114, 1116-24 (1973)
76. Nurse P., Zavitz K. H. & K. J. Marians: Inactivation of the *Escherichia coli* priA DNA replication protein induces the SOS response. *J Bacteriol* 173, 6686-93 (1991)
77. Town C. D., Smith K. C. & H. S. Kaplan: DNA polymerase required for rapid repair of x-ray--induced DNA strand breaks in vivo. *Science* 172, 851-4 (1971)
78. Paterson M. C., Boyle J. M. & Setlow R. B. Ultraviolet- and X-ray-induced responses of a deoxyribonucleic acid polymerase-deficient mutant of *Escherichia coli*. *J Bacteriol* 107, 61-7 (1971)
79. Kogoma T., Cadwell G. W., Barnard K. G. & T. Asai: The DNA replication priming protein, PriA, is required for homologous recombination and double-strand break repair. *J Bacteriol* 178, 1258-64. (1996)
80. Ennis D. G., Amundsen S. K. & G. R. Smith: Genetic functions promoting homologous recombination in *Escherichia coli*: a study of inversions in phage lambda. *Genetics* 115, 11-24 (1987)
81. Zieg J., Maples V. F. & S. R. Kushner: Recombinant levels of *Escherichia coli* K-12 mutants deficient in various replication, recombination, or repair genes. *J Bacteriol* 134, 958-66 (1978)
82. E. B. Konrad: Method for the isolation of *Escherichia coli* mutants with enhanced recombination between chromosomal duplications. *J Bacteriol* 130, 167-72 (1977)
83. A. Kuzminov: Collapse and repair of replication forks in Escherichia coli. *Mol Microbiol* 16, 373-84 (1995)

Key Words: DNA repair, Replication block, Pol I, Stable DNA replication, transcription-coupled repair, R-loop, polA, Polymerase, Review

Send correspondence to: Dr Lawrence A. Loeb, Department of Pathology, Box 357705, HSB K056, Seattle, WA 98195-7705, Tel: 206-543-6015, Fax 206-543-3967, E-mail: larryloeb@earthlink.net

http://www.bioscience.org/current/vol10.htm