CRITICAL ROLE OF R-LOOPS IN PROCESSING REPLICATION BLOCKS

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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 reassemled 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 play a 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 CoIE1 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 CoIE1 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 mutagenesis”), 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
R loop-dependent fork assembly

Table 1. Most frequently reported Pol A mutations

<table>
<thead>
<tr>
<th>Allele</th>
<th>Mutation</th>
<th>Amino Acid substitution</th>
<th>Location</th>
<th>Ref.</th>
<th>5'→3' exo activity</th>
<th>Polymerase activity</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>polA1</td>
<td>G(1025)→A</td>
<td>W(342)→Am</td>
<td>N-terminal end of polymerase</td>
<td>62</td>
<td>Normal</td>
<td>Low levels</td>
<td>20</td>
</tr>
<tr>
<td>resA1</td>
<td>C(892)→T</td>
<td>Q(286)→Am</td>
<td>C-terminal end of 5' nuclease</td>
<td>62</td>
<td>Normal</td>
<td>Low levels</td>
<td>63</td>
</tr>
<tr>
<td>polA12</td>
<td>G(1631)→A</td>
<td>G(544)→D</td>
<td>Motif 1 in polymerase domain</td>
<td></td>
<td>Normal</td>
<td>Temperature-sensitive</td>
<td>20</td>
</tr>
<tr>
<td>polA2099::Tn</td>
<td>Tn insertion, duplicating base pairs 2004 through 2612</td>
<td>Deletion of last 58 amino acids</td>
<td>64</td>
<td>Normal</td>
<td>Low</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>polA107</td>
<td>A(230)→G</td>
<td>Y(77)→C</td>
<td>5' nuclease domain</td>
<td>62</td>
<td>Low levels</td>
<td>normal</td>
<td>65</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Increased Replication Block</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>rnhA 1 polA12</td>
<td>24</td>
</tr>
<tr>
<td>recA441 polA12</td>
<td>44</td>
</tr>
<tr>
<td>recA730 polA12</td>
<td></td>
</tr>
<tr>
<td>rec718 polA1, polA12</td>
<td>53</td>
</tr>
<tr>
<td>Impaired Fork Rescue</td>
<td>Ref.</td>
</tr>
<tr>
<td>priA polA12</td>
<td>67</td>
</tr>
<tr>
<td>recG polA1, polA12</td>
<td>25</td>
</tr>
<tr>
<td>recA polA1, polA12</td>
<td>68,69</td>
</tr>
<tr>
<td>recBC polA12</td>
<td>70</td>
</tr>
<tr>
<td>rvuAB polA12</td>
<td>30</td>
</tr>
</tbody>
</table>

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). 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

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

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 rifampycin, whereas initiation at D-loops is rifampycin-resistant. In one study, which used thymidine starvation to induce SOS, ΔpolA cells showed a clear inhibition of rifampycin-sensitive SDR (21). This contradicts an earlier study showing no effect on rifampycin-sensitive SDR, but those results need to be regarded with caution because SOS was induced by UV irradiation, which has notoriously pleiotropic effects (22). Rifampycin-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 rifampycin-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). PolI 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 defined 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 (rpoB*) 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 CoE1 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 CoE1 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 CoE1 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 recA718 cells have been complemented by a variety of polymerases lacking 5'-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 (Tag) polymerase (53-56). In all cases except Tag, complementation correlates with restoring ability of polA12 recA718 cells to support CoE1 plasmid replication at the restrictive temperature, which further supports the proposition that extension of an RNA primer is limiting in polA12 recA718 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 CoE1 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 CoE1 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

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10. REFERENCES

R loop-dependent fork assembly

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