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Review

Roadworks of DNA Damage Bypass during and after Replication

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Duplication of the genome must be faithfully carried out in proliferating cells. DNA replication is the stage in which DNA damage becomes truly dangerous and potentially causes cell death or genomic instability. DNA damage bypass mechanisms have evolved as the 'last minute' processes to protect the quality of genome replication from these risks. Damage bypass provides a highly flexible mechanism to tolerate various types of DNA damage during replication. Recent studies have highlighted that bypass mechanisms can be uncoupled from global genome replication: i.e., the time of action of DNA damage bypass is not fixed at a particular point during genome replication. Although DNA damage bypass mechanisms are conserved throughout organisms, their regulation is different between prokaryotes and eukaryotes. On one hand, the bypass mechanisms of prokaryotes are mainly dependent on upregulation of transcripts under the SOS regulon. On the other hand, in eukaryotes, DNA damage bypass is activated by ubiquitylation of the replication sliding clamp, PCNA. This review starts with our understanding of the basis of lesion-bypassing mechanisms in the bacterial system, advances to recent views of the molecular mechanisms underlying eukaryotic DNA damage bypass and specifically focuses on how the bypassing mechanisms provide temporally and structurally flexible functions.

Key words: DNA damage bypass, PCNA ubiquitylation, SOS response, translesion DNA synthesis, template switching, mutagenesis, post-replication repair, DNA replication, genomic instability

Introduction

Duplication of the genome is the means of transmission of the genetic code from one generation to the next. Its accuracy and efficiency is one of the most impressive feats of biology. What factors are required for such a skillful process? Certainly, the replication machinery is a highly sophisticated apparatus that can provide efficient duplication of the genome during a limited time within the cell cycle. However, the road of replication is not always flat and the removal of roadblocks is necessary for accurate replication. Such roadblocks include the modification of DNA structure due to chemical or physical change; i.e., the formation of DNA damage. To cope with such obstacles, all the organisms are equipped with DNA repair and DNA damage bypass pathways.

Environmental factors (UV, ionizing radiation, chemical agent, etc.) and endogenous factors (reactive oxygen species, enzymatic reaction, etc.) all can potentially damage DNA. Some base lesions cause the replicative polymerases to incorporate incorrect nucleotides, generating point mutations; however most lesions inhibit DNA replication directly by blocking the polymerase reaction. To minimize these effects, DNA repair pathways such as nucleotide excision repair (NER) and base excision repair (BER) can eliminate the damage before DNA replication. However, it is inevitable that some lesions escape these repair pathways and are thus presented to the replication machinery. The remaining DNA lesions can potentially cause prolonged replication stalling and/or replication fork collapse. If these are unresolved, the result can be cell death or genomic instability. To prevent these catastrophic events, DNA damage bypass pathways are employed to facilitate replication over DNA lesions. In this sense, DNA damage bypass works as a ''last resort'' to ensure the completion of replication in the presence of replication-blocking lesions.

What are the mechanisms used to bypass polymerase-blocking lesions? First, specialized DNA polymerases can incorporate nucleotides opposite DNA lesions. Thus, translesion synthesis (TLS) can extend DNA across polymerase-blocking lesions such that the replicative DNA polymerases can subsequently continue synthesis (Fig. 1-iv and vi). TLS polymerases are endowed with catalytic sites that can readily accommodate various lesions on the template strand (1). However, the lesion-bypassing feature comes at the expense of increased misincorporation rates and poor processivity on

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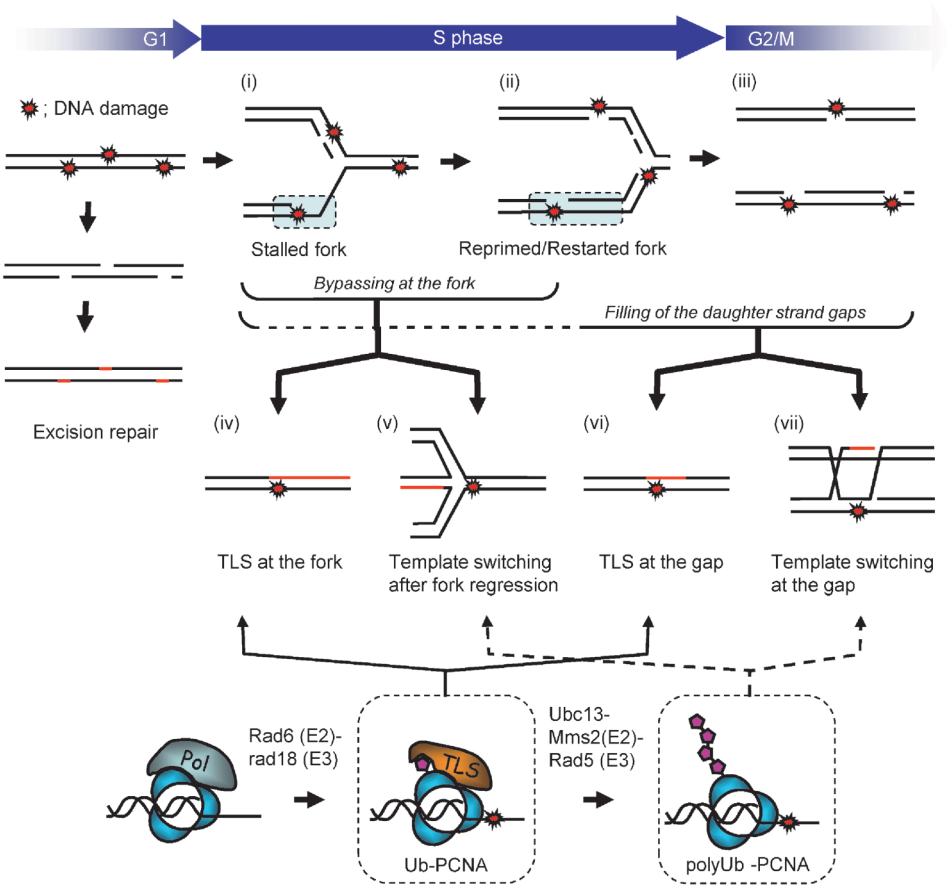

Fig. 1. Representation of DNA damage bypass mechanisms within and after S phase and the involvement of PCNA ubiquitylation in translesion synthesis and error-free bypass pathways. DNA synthesis via a bypass mechanism is highlighted as a red line.

undamaged DNA. The second mechanism of lesion bypass exploits the presence of two sister duplexes during replication: following the arrest of DNA synthesis by a lesion, replication is resumed on the sister strand as an alternative template DNA, a process mediated by strand exchange which is catalyzed by recombination enzymes (Fig. 1-v and vii). As this recombination sub-pathway avoids using the damaged base as a template, this is known as an ''error-free'' mechanism and has advantages for the accuracy of genome replication.

Studies of DNA damage bypass started with the development of a method to examine the size of newly-synthesized strands in UV-irradiated cells, which was initially used in *E. coli* by Rupp and Howard-Flanders (2). They demonstrated that, in NER-deficient cells, UV-induced damage caused discontinuities in the newly-synthesized strands without significantly delaying overall DNA synthesis. This implied the existence of daughter strand gaps at the end of replication. Importantly, the spacing of these discontinuities was similar to those of the initial UV-induced lesions on the parental DNA, suggesting the presence of lesions opposite the daughter strand gaps (Fig. 1-iii). Thus, 'post-replication repair' (PRR) was defined as the repair of these gaps. The meaning of the term ''DNA damage bypass'' is related to that of PRR; but PRR more precisely describes the events underlying the observations made using the traditional method of Rupp and Howard-Flanders: this generally detects gap filling events after the bulk of replication has completed. PRR has also been later characterized in eukaryotes and this traditional assay continues to be a valuable tool in studies in DNA damage bypass (3–5).

Subsequently, work in bacterial and eukaryotic model systems has identified many factors involved in DNA damage bypass and has provided insights into the details of the underlying pathways as well as their regulation. As a consequence of extensive work in a variety of systems, models describing DNA damage bypass have become highly diverse. In particular, the timing and targets of lesion-bypassing activities have represented the controversial topics. Because PRR-related proteins appear to be functional when replication forks are stalled by polymerase-blocking lesions, many models of DNA damage bypass have tended to focus on replication fork-associated mechanisms (Fig. 1-i). However, more

recent studies have highlighted the role of PRR mechanisms using new techniques and suggest that PRR can be uncoupled from direct association with the fork. Here I review the mechanisms of DNA damage bypass pathways and then, incorporating our recent knowledge of replication, examine the current models.

DNA Damage Bypass in Prokaryotes; 'SOS' Signals Rescue Cells in Danger

Following the documentation of PRR, studies in *E. coli* identified two basic lesion bypassing pathways consisting of either recombination-based or TLS mechanisms. With regard to their regulation, the SOS regulon plays a crucial role in the control of transcription of genes relevant to DNA repair and DNA damage bypass. Under the control of the SOS system, expression of targeted genes in unperturbed cells is negatively regulated by the LexA repressor. Upon exposure to genotoxic stress, polymerase blockage at DNA lesions generates ssDNA stretches which become coated with RecA filament. This filament stimulates autoproteolytic cleavage of LexA, thereby permitting transcription of the target genes, collectively known as the SOS regulon. The fact that the non-inducible *lexA* mutant confers a defect in PRR and UV-induced mutagenesis confirms the crucial role of the SOS response as a regulatory element of DNA damage bypass.

Prior to the discovery of the SOS response, the recombination pathway was found to be a key mechanism underlying PRR, playing a predominant role in bacterial cells (6,7). RecA is, in addition to its role in the SOS response, necessary for all recombination events in bacterial cells. When a replication polymerase stalls in bacteria, it can be restarted just downstream of the lesion. RecA filaments form on resulting ssDNA stretches ''daughter strand gaps'' (Fig. 1-iii). This is assisted by the RecFOR complex and these RecA-nucleoprotein filaments promote strand exchange. Following strand exchange, gap repair is completed by DNA synthesis using the sister strand as a template and the resulting joint molecule, Holliday junction, (Fig. 1-vii) is resolved by the RuvABC complex (8,9). In addition to PRR activity at daughter strand gaps, the recombination pathway is thought to be involved in the restart of stalled replication forks (10,11). In this process, fork regression, which involves the re-pairing of the DNA template strands such that the fork is moved backwards, has been proposed as a mechanism to promote bypass of polymerase blocking lesions: the newly-synthesized strands are paired to generate a Holliday junction with one short branch (Fig. 1-v), which is often termed a 'chicken foot' structure, which enables DNA synthesis by the use of the other nascent strand as a template. The simplest path to fork regression is non-enzymatic; the positive supercoiling that builds up ahead of a fork will lead to spontaneous fork regression. The stalled replication fork can also be enzymatically branch migrated by RecG helicase, which tolerates the ssDNA gap located at the junction of the substrate DNA. An alternative mechanism would be that a RecA filament is formed on the extended ssDNA stretch generated between the fork and the site of polymerase-blockage (Fig. 1-i) which then promotes annealing of the leading strand and lagging strand templates, which will also result in fork regression. RecA and RecFOR proteins have also been reported to stabilize the intermediates of unwound replication forks (12). Taken together, recombination mechanisms provide multiple layers of DNA damage-bypassing pathways. Considering that 50% of *recA*-deficient cells are dead under normal condition, replication restart mechanisms must be routinely active (13). Under more severe genomic stress, the SOS response induces expression of recombination proteins such as RecA and RecF to promote further events; e.g., PRR.

TLS also contributes to DNA damage tolerance in *E. coli* by allowing direct bypass of DNA lesions but to a lesser extent than the recombination pathway. In *E. coli*, five DNA polymerases have been identified, PolIII and PolI are required for genome replication, while the other three polymerases PolII, PolIV and PolV play roles in TLS (14,15). In particular, PolIV and PolV are categorized as Y-family DNA polymerases (16), whose structures are apparently specialized for DNA damage tolerance. PolII, PolIV and PolV are encoded by *polB*, *dinB* and *umuC/umuD* genes respectively and are all regulated within the SOS regulon. However, only cells defective for PolV exhibits moderate sensitivity to UV light, indicating that PolII and PolIV are more likely to play less important roles in TLS and have alternative functions. For example, PolIV is important for untargeted mutagenesis that occurs at undamaged sites on the DNA (17), which is suggested to be a deliberate method to induce genetic variation, so-called adaptive mutagenesis (18).

The PolV-encoding *umuC* and *umuD* genes were initially isolated as being responsible for UV-induced mutagenesis. In common with other Y-family polymerases, PolV functions with both lower fidelity and broader substrate specificity, allowing replication over damaged templates. In addition to being part of the SOS regulon, autoproteolysis of the $UmuD_2$ subunit to $UmuD'_2$ is caused by interaction with the RecA filament to form active PolV ($UmuD'_2C$), rendering a delay of PolV function compared to other members of the SOS regulon (14). Furthermore, the catalytic activation of PolV occurs *in trans* by RecA filaments, i.e., by fillament present on separate ssDNA molecules from the strand on which PolV becomes active (19). These exquisite and multiple steps regulating the activation of PolV appear to restrict error-prone TLS to the right place and

right time when otherwise overwhelming DNA damage needs to be bypassed. Under these circumstances, the risk of a high mutational load is tolerated because of the gain in relative fitness.

In summary, the SOS regulon is an elegant example of the strategies underlying the regulation of DNA damage bypass. Fine tuned induction of gene function composes the following elements; first, a damage-inducible feature to avoid unnecessary action of the low fidelity mechanisms, second, temporally limited activities of error-prone pathways, such as PolV-dependent TLS, and third, simplicity–allowing for an efficient and rapid response to DNA damage. Although the regulatory mechanisms in eukaryotes are distinct from those characterized in prokaryotes, close regulation is a key common feature.

DNA Damage Bypass in Eukaryotes; The Master Controller Is PCNA Ubiquitylation

In parallel to studies using *E. coli*, genetic work using budding yeast has identified many genes involved in DNA repair and DNA damage bypass. For example, the *RAD* genes are defined by mutants sensitive to UV-light and/or ionizing radiation (20), whereas the *REV* genes are defined by mutants that exhibit significantly reduced UV-induced mutagenesis (21). Epistasis analysis using these mutants classified the corresponding genes into a few epistasis groups. Among them, the *RAD6* epistasis group has been described as the one involved in damage-induced mutagenesis and PRR. The *RAD6* group was later categorized into three pathways (5,22,23). Two of these pathways involve TLS and are distinguished based on their mutagenic properties. The first requires polymerase η (Polη, encoded by *RAD30*, the yeast homolog of xeroderma pigmentosum variant gene XPV) and the second requires polymerase ζ (Polζ, encoded by *REV3* and *REV7*). The third pathway, named error-free damage avoidance, was later demonstrated to involve template switching events between sister chromosomes (Fig. 1-v and vii) (24).

These three pathways are conceptually reminiscent of those discovered in *E. coli*, although the identities and function of many proteins required for their function are not directly related to the lesion bypass mechanisms characterized in bacterial cells. Many of the *RAD6*-group genes, including *RAD6*, *RAD18*, *RAD5*, *UBC13* and *MMS2*, encode ubiquitylation enzymes (25). Hoege *et al.* showed that these ubiquitylation proteins target the replicative sliding clamp complex, PCNA, that functions as a key binding platform for a wide variety of replication enzymes (Fig. 1. bottom) (26). In response to DNA damage, PCNA is mono-ubiquitylated by the E2-E3 enzyme complex, in which the Rad6 ubiquitin-conjugating (E2) enzyme and the Rad18 E3 ligase bind tightly to each other. The mono-ubiquitylated form of PCNA can subsequently be poly-ubiquitylated through lysine 63 linkages of ubiquitin by the Ubc13/Mms2/Rad5 complex (Ubc13/Mms2; E2 complex, Rad5; E3 ligase). Genetic analysis supports a model whereby mono-ubiquitylated PCNA specifically mediates TLS pathways, whereas poly-ubiquitylated forms of PCNA are required for the switch to error-free damage avoidance (27) (depicted in Fig. 1). *In vitro* reconstitution using recombinant yeast proteins demonstrated sequential but independent actions of Rad6-Rad18 and Ubc13/Mms2-Rad5 complexes and the stepwise manner by which K63-linked poly-ubiquitin chains form on PCNA (28). PCNA ubiquitylation exists in all characterized eukaryotic systems (29–33). In mammalian cells, the homologs of the Rad6 E2 enzyme and the Rad5 E3 ligase are duplicated as RAD6A/RAD6B and SHPRH/HLTF (34,35). Interestingly, the human homolog of Rad18 and the SHPRH protein are both reported to bind to TLS polymerases and are proposed to act as chaperones to guide the polymerases to sites of damaged DNA (36–38). Thus, the roles of ubiquitin enzymes in mammalian cells are likely to be diverse and PCNA modification may be regulated differently (also see reviews (39,40)).

How PCNA modification mediates the lesion-bypass has become a topic of great interest. An interaction between human TLS polymerase Polη and mono-ubiquitylated PCNA was identified and shown to be required for damage-induced localization of Polη (29,30). This interaction occurs through the affinity of the ubiquitin-binding domain of Polη for ubiquitin (41). In eukaryotes, four Y-family polymerases; Rev1, Polη, Polκ, Polι and one of B-family polymerase; Polζ are recognized as TLS polymerases, based on their physiological roles (note: not all eukaryotes possess all these TLS polymerases: for example, budding yeast is missing Polκ and Polι, and fission yeast is missing Polι) (42,43). All Y-family polymerases have ubiquitin-binding domains, whereas the B-family polymerase, polζ, does not (42). However, it appear to be controversial whether the catalytic activity at sites of DNA damage for TLS polymerases is enhanced by modified PCNA (44,45). In addition, interactions between the different polymerases have been reported to be important for their regulation; in particular, Rev1 is suggested to function primarily as a scaffold protein to localize other TLS proteins (46–48). Such interactions were initially proposed to contribute to the choice of polymerases for the insertion of nucleotides opposite specific based damages. More recently, their importance has been explained by way of a 'two-polymerase' mechanism. In this model, the insertion of nucleotides opposite to lesions and extension from the lesion-nucleotide pair are performed by separate polymerases. Rev1 may, therefore, have a role in bridging between two polymerases (49,50). The interaction be-

tween Rev1 and Polζ has been particularly well characterized: in early work using yeast genetics, Rev1 was categorized into the error-prone TLS pathway together with Rev3 and Rev7 that together encode Polζ (51). Consistent with this, Rev1 enhances Polζ activity for extension on a damaged template (52) *in vitro* and the Rev1-Polζ interaction is conserved in all characterized eukaryotic organisms (53). The unique role of Polζ as the 'extender' polymerase in the two-polymerase model might require a tight association with Rev1. It should be also noted that some TLS polymerases have roles apart from DNA damage bypass. Specifically, Polκ is characterized as a factor for NER in stationary cells (54) and has been demonstrated to have potentially negative effects on DNA replication (55).

The requirement for poly-ubiquitylated PCNA in the error-free bypass mechanism was confirmed in several studies in budding yeast (24,56,57). However, the molecular mechanisms underlying this sub-pathway of PRR are largely unknown. In addition to its essential function for PCNA poly-ubiquitylation, the Rad5 E3 ligase also harbours an ATPase domain that confers DNA helicase activity. Assays using mutants where the ATPase or the E3 ligase activities are separately abolished indicated that these activities function independently but need to be coordinated together for template switching events to occur efficiently (58,59). As no binding partner for poly-ubiquitylated PCNA has been identified, proposed mechanisms suggest that poly-ubiquitylation of PCNA disrupts the association of PCNA-bound replication proteins or destabilizes the replication machinery, which could be inhibitory to template switching reactions. *In vitro* data demonstrated that the helicase activity of Rad5 is highly specialized to act on replication forks (Fig. 1-i) and does so without exposing extended single-stranded regions (60). Thus it has been suggested that unwinding of stalled replication forks catalyzed by Rad5 promotes the regression of replication forks associated with the pairing of the nascent leading and lagging strands (chicken foot formation), thereby enabling template switching reactions through use the nascent sister strand as the template DNA (Fig. 1-i to v). This mechanism would be analogous to the *E. coli* RecG-dependent fork regression described above. The promotion of the template switch events by the helicase activity of Rad5 could be assisted by combining it with the dissociation of proteins from PCNA due to poly-ubiquitylation of PCNA; thereby, generating the space for template switching at the replication fork. This scenario could account for the restart of lesion-stalled forks. However, it would appear to be less relevant for the situation of gap-filling by PRR after the fork has passed by (Fig. 1-iii to vii) (56). Indeed, experiments using 2D gel analysis to resolve DNA structures formed *in vivo* showed the accumulation of sister chromatid junctions in a Rad5-dependent manner, which appear to be intermediates of gap-filling that occurs behind the replication fork, rather than regressed replication forks themselves (57,58).

What Triggers PCNA Ubiquitylation?

Given the significance of the regulatory role of PCNA ubiquitylation, how PCNA ubiquitylation is elicited by DNA damage is an important issue. To cope with the many types of DNA damage a cell encounters, the regulatory elements of PCNA ubiquitylation are expected to sense a common feature consequent on DNA damage. Comparison of the levels of PCNA modification induced by many different types of DNA damage revealed that high levels of PCNA ubiquitylation are caused by replication-blocking lesions, the target of lesion bypassing pathways. Arrest in S-phase with hydroxyurea, which causes replication fork stalling by nucleotide depletion, is also sufficient to trigger PCNA ubiquitylation, suggesting that cellular states and/or replicative structures during S phase are the primary signals for PCNA ubiquitylation. Consistent with this, in replicating *Xenopus* egg extracts, ssDNA generated by the uncoupling of leading-strand DNA polymerase from the replication forks triggers mono- and di-ubiquitylation of PCNA (Fig. 1-i) (61). Additionally, a requirement of ssDNA-binding replication protein A (RPA) for mono-ubiquitylation of PCNA was demonstrated in both budding yeast and mammalian cells (62,63). The Rad18 E3 ligase physically interacts with RPA (62).

Taken together, the results described above led to a model whereby the Rad6-Rad18 complex is recruited to extended ssDNA stretches via an association with RPA, thereby promoting mono-ubiquitylation of PCNA molecules at the proximal primer-terminus. Thus, the regulatory requirement of PCNA ubiquitylation arises locally at the site of DNA damage and hence does not cause transactivation of low fidelity events at inappropriate undamaged sites. Perhaps PCNA ubiquitylation is a more effective solution to minimizing the risk of genomic instability than the transcriptional control such as is seen for the bacterial SOS response. In addition, deubiquitylation of PCNA plays an important role in preventing unnecessary actions of error-prone mechanisms (64,65).

Coupling and Uncoupling of DNA Damage Bypass from Genome Replication

Since the discovery of the lesion bypass activity of Y family DNA polymerases and following the intensive studies on the role of recombination at replication forks in *E. coli*, many studies of DNA damage bypass have focused on the events occurring directly at the blocked replication fork. A prime consideration for influence of DNA lesion at the arrested fork is the continuous versus

discontinuous nature of leading and lagging strand synthesis. While reinitiation downstream from the lesion by Okazaki fragment synthesis permits replication on lagging strands, the continuous nature of DNA synthesis on the leading strand leads to the expectation that a single site of damage would disrupt the replicative machinery in eukaryotic cells (Fig. 1-i) (66,67). Thus, a prolonged arrest of leading strand DNA synthesis would lead to uncoupling of DNA polymerase from the replicative helicase (68–70) which further predicts that lesions on the leading strand inhibit DNA synthesis to a greater extent. Such considerations have led to the proposal that lesion-bypassing pathways have a primarily role in contribute to the in-situ resolution of arrested replication forks.

In prokaryotes, the experiments of Rupp and Howard-Flanders, which assayed daughter strand DNA synthesis in the presence of UV-induced damage, clearly indicated that a polymerase-blocking lesion does not halt bulk DNA synthesis upstream downstream of the lesions, as depicted in Fig. 1-ii and iii (2). This is consistent with the idea that, by skipping over DNA lesions on both the leading and lagging strands, ssDNA gaps can be left opposite the lesions on either daughter strand and these gaps can be filled after the bulk of replication is complete (i.e., 'post'-replication repair as originally defined) (Fig. 1-vi and vii). Later work in budding yeast and mammalian cells also demonstrated similar PRR phenomena (3–5,71). Because of this accumulated evidence, some reviews are critical of focusing only on mechanistic descriptions of the direct restart of lesion-arrested replication forks (72,73). Consistent with a classical PRR model, electron microscopic image by Lopes *et al.* revealed that UV irradiation causes ssDNA gaps in the vicinity of replication forks (74) and that these gaps exist on both the leading strand as well as lagging strand (Fig. 1-ii). This visual evidence of discontinuities on the leading strand provides a seminal observation that may reconcile the role of PRR in lesion-bypass with the observation that bulk DNA replication is not appreciably slowed by UV irradiation despite the apparent fragmentation of the nascent strands in Rupp and Howard-Flanders' style experiments. However, a clear demonstration of leading strand repriming remains to be made in eukaryotes (this topic is discussed in the next section).

Two recent studies in budding yeast particularly focused on the timing of DNA damage bypass in the course of replication. Karras *et al.* designed PRR proteins fused with the regulatory element of the mitotic cyclin Clb2 that limited their presence and thus function to G2/M phase (75). Using an alternate approach, Daigaku *et al.* (76). controlled the timing of PCNA ubiquitylation by regulating the *RAD18* gene under the control of inducible promoters (the *GAL* promoter and *tet*-regulated promoter). Both these independent studies indicated that TLS and error-free bypass are fully operational after bulk DNA replication has been completed. When PRR was uncoupled from genomic replication, incorporation of the thymine analog BrdU by PRR pathways was observed to be restricted to small patches that were widely dispersed along the chromosomes (76). This pattern is clearly distinct from that derived from bulk replication, where nucleotides are incorporated in longer stretches. Importantly, the numbers of PRR patches were dependent on the dose of UV given, providing an estimation that 20% to 30% of lesions result in daughter strand gaps. Accordingly, the filling of daughter strand gaps after bulk DNA replication likely accounts for a significant fraction of DNA damage bypass (Fig. 1-i, ii and iii). Finally, the existence of ssDNA gaps was shown to contribute to checkpoint activation, resulting in a cell cycle arrest at the G2/M boundary (75–77). This checkpoint-dependent cell cycle arrest presumably ensures that lesion bypass is completed before entry into mitosis.

In support of the postreplicative nature of DNA damage bypass, the levels of yeast Rev1, which is regulated by proteasomal degradation, is high in G2/M cells (78,79). This possibly reflects that the TLS mechanism targets ssDNA gaps after bulk DNA replication. Further data supporting a post-replicative role for PRR pathways comes from recent work in human cells transfected with plasmids containing specific lesions. This showed that the mutagenic signatures of TLS are remarkably different between S and G2 phase, despite the similar extent of overall TLS events (80). Some hotspot mutations that are dependent on Rev1/Polζ appear to be more prevalent in G2 phase and therefore may be due to the activation of error-prone TLS during G2 phase. Presumably, the activation of error-prone TLS is limited until gap-filling is actually required. This may explain the higher mutation rates reported for both human cells (81) and budding yeast (82) at late replication times, when cells are closer to G2.

The accumulated data demonstrate that lesion-bypassing mechanisms can be uncoupled from genomic replication; however, this notion does not exclude the activity of DNA damage bypass during the time when genomic replication is actively ongoing. Damage-induced PCNA ubiquitylation is usually maximal during active replication (62,76), suggesting that lesion-bypassing can be initiated during S phase. In several model systems and in proliferating mammalian cell lines, PCNA ubiquitylation is also observed during unperturbed S phase (31–33,64). In budding yeast, consistent with the strong signal of PCNA ubiquitylation during S phase after genotoxic treatment, a defect in lesion bypass causes slower progression of S phase in the presence of DNA damage and, at the same time, activation of the

DNA damage checkpoint is enhanced (76,83). This phenomenon indicates that PRR pathways normally do contribute to damage processing during S phase, thereby effectively preventing checkpoint signalling during replication. However, these results do not establish if the slower replication in lesion bypass-deficient cells is due to checkpoint-mediated effects or due to direct inhibition of DNA replication by blocking lesions.

In addition to the observation of these trends during S phase, there are also genetic interactions that are indicative of the relationship between lesion bypass mechanisms and replication ensembles: when the function of Polδ the replicative polymerase that functions in lagging strand DNA synthesis, is partially compromised, the error-free bypass pathway is detrimental to cell growth and viability (84). This suggests that ubiquitylated PCNA itself, or the template switching mechanism, have the potential to interfere with the replication machinery during S phase. Furthermore, budding yeast Rev1 can interact simultaneously with Pol32 (a non-essential subunit of yeast Polδ: mammalian homolog; p66 and *S. pombe* homolog; Cdc27) and Polζ (85). The domain of Rev1 for these interactions is prerequisite for UV-induced mutagenesis and hence for Polζ function during TLS. This suggests a link between TLS and the replicative machinery via Rev1. However, it remains to be seen whether Polδ recruits TLS factors to assist the resumption of stalled replication forks or, alternatively, if Rev1 involves Polδ components as additional factors for TLS.

In summary, the nature of DNA damage bypass within and after S phase may encompass two potential modes of action: first, DNA damage bypass occurs at arrested replication forks when the leading-strand polymerase encounters DNA lesions (Fig. 1-i and ii). Second, daughter strand gaps generated behind replication forks are filled in post DNA replication, namely PRR (Fig. 1-iii) (See also the review (86)). Alternatively, it could also be considered that most polymerase blockages are efficiently overcome by rapid repriming downstream of the lesion and consequently daughter strand gaps arise promptly and persist during S phase (the structure shown in Fig. 1-ii). In this scenario, PRR-like bypass events could also be occurring during S phase, but happen after the fork has resumed. In this case, the efficient formation of daughter strand gaps behind replication forks would be postulated to cause the accumulation of ubiquitylated PCNA, that is loaded and modified at the primer terminus. This would be consistent with the persistence of PCNA ubiquitylation after the completion of S phase (63,75,76). Finally, it should be noted that the regulatory role of PCNA ubiquitylation could be more complex and/or may be distinct among organisms. For example, in the chicken B cell line DT40, PCNA ubiquitylation is essential for the PRR mechanism after S phase but is not required for the bypass of DNA damage at ongoing replication forks (87). Cellular factors may vary depending on cell-type and organism, reflecting the level of cell proliferation, the activities of specific ubiquitination enzyme, etc.

Replication Ensembles Underlying PRR

The data discussed above provides insights into physiological responses to replication-blocking lesions and the pathways that ensure the completion of genome replication. In addition, the behaviour of the replication machinery when it encounters DNA damage is expected to play a key role in regulating DNA damage bypass. One controversial issue is the mechanism by which the replisome skips over polymerase-blocking lesions, leading to the generation of daughter strand gaps behind the fork. As mentioned above, an encounter between a DNA polymerase and a DNA lesion will have different consequences depending on which strand contains the damage. The nature of lagging strand DNA synthesis permits synthesis of next Okazaki fragments ahead of a polymerase-blocking lesion, and a ssDNA gap will therefore be generated opposite the lesion. In contrast, the continuous synthesis of the leading strand does not *de facto* allow for the formation of daughter strand gaps. However, evidence for such replication fork-associated gaps was observed on both leading and lagging strands in UV-irradiated yeast cells using electron microscopy (74). Reconstitution studies of replication forks using the *E. coli* replisome potentially sheds light on discontinuities during leading strand synthesis and provided evidence for repriming events downstream of the blocking lesion at least in prokaryotes. Surprisingly, in this system, the repriming event can occur without the additional loading of replisome components (i.e., without the action of PriA) following an encounter with a defined lesion (Fig. 1-ii). This suggests that, during normal replication, the replisome has the ability to skip over lesions (88). Furthermore, even when the replisome is dissociated from the stalled fork, all the components required for a repriming event and subsequent resumption of DNA synthesis could be re-loaded in a PriC-dependent manner (89) (PriC is the loading factor for the replicative apparatus and is active at the ssDNA stretch associated with the fork structure: see review (90) for further details). An intrinsic ability to reprime the leading strand replication machinery could be considered as an important mechanism that acts to minimize any delay to replication due to DNA lesions. The consequences would be the formation of a gap opposite the lesion that would be a substrate for PRR events on the leading strand (defined as gap filling, as oppose to lesion bypass by the replisome). However, as stated before, direct repriming events in eukaryotes have yet been identified neither *in vitro* nor *in vivo*. Whether the eu-

karyotic replisome can behave similarly to those of prokaryotes is clearly of great interest.

In *E. coli*, OriC is the only replication origin. Because replication is deliberately terminated at Ter sites approximately equidistant from OriC, any location in the *E. coli* genome must be replicated in a unidirectional manner. Therefore, repriming events are theoretically the only means to restart DNA synthesis without bypassing polymerase-blocking lesions. Conversely, in eukaryotes, as a large number of origins are initiated on a single chromosome to duplicate the DNA, the initiation of neighbouring origins can potentially compensate for an arrested fork. Replication origin features vary depending on the organism. In budding yeast, replication origins have a common consensus sequence, whereas, in fission yeast and metazoans, origins do not share a consensus sequence. Instead, the origin firing in these organisms has more stochastic features, depending on sequence content, the status of transcription, chromatin structure, etc. (for a detailed review, see (91)). Under conditions of replication stress, origin initiation is actively regulated in eukaryotes; activation of the S phase checkpoint limits late origin firing. However, the regulation of origin initiation becomes more complicated with increased stochastic nature; studies in mammalian cells reported that a set of dormant origins proximal to active replication machineries are activated under replication stress (92–94). Thus, it could be suggested that flexibility of origin firing avoids the accumulation of unreplicated regions caused by polymerase-blocking lesions. When a fork arrested by a lesion is approached by a second active fork, a small ssDNA gap opposite the lesion would remain between the two nascent strands, i.e., opposite the damage template. However, it should be noted that the control of origin firing has been characterized under conditions in which replication is very strongly inhibited by hydroxyurea (nucleotide depletion) or aphidicolin (DNA polymerase inhibitor) and that the effect of replication-blocking base lesions is largely unknown.

Do DNA Damage Bypass Pathways Have Differentiated Roles?

TLS bypasses replication-blocking lesions and is confined to one of the sister duplex strands. Conversely, error-free bypass pathways involve both sister strands to allow a template switching mechanism, a relatively more complex reaction. Defects of TLS and error-free bypass pathways yield comparable sensitivities to UV-light in budding yeast and many experiments closely examined the contribution of both pathways in the presence of various types of DNA damage (86). However, the relative contribution of each of these tolerance pathways in eukaryotes is not precisely known due to the scarcity of appropriate assays. In reality, depending on the type of experimental system, the importance of TLS and error-free bypass is likely to be quite variable (86). This may be attributed to the different requirement of the two mechanistically distinct pathways. One suggestion is that they operate at different times in the cell cycle. However, it remains unclear what the important criteria are for the choice between these pathways.

TLS and error-free bypass pathways could have different preferred structures/DNA damage as targets. This is certainly the case within the TLS pathway(s) because each TLS polymerase has a distinct ability to promote synthesis depending on the type of DNA damage. For example, for UV-induced lesions, the T-T cyclobutane pyrimidine dimer (CPD) is readily bypassed by Polη in an error-free manner. In contrast, the ability of any of the TLS polymerases to bypass a T-T 6,4 photoproduct (6,4 PP) is much less efficient because of the large structural distortion caused by this particular lesion. Gibbs *et al.* estimated, in wild-type yeast cells, a 60% chance of TLS bypass for a CPD lesion compared to a 3% chance of bypass for a 6,4 PP lesion (95,96). Since TLS promotes bypass of only a fraction of 6,4 PP, error-free template switching would appear to be the critical mean of bypassing this specific lesion. Consistent with this, gap repair opposite a 6,4-PP on a plasmid is largely the consequence of template switching between sister strands (24). The inability of TLS to bypass 6,4-PPs could also cause persistent daughter strand gaps opposite this lesion, which would be consistent with the primary contribution of error-free pathways to PRR activity in UV-irradiated cells (56). Accordingly, bypass mechanisms following UV-light are likely to require cooperation between bypassing pathways because replication is challenged by multiple types of lesions. The mechanistic simplicity of TLS may provide a means of rapid and efficient bypassing at non-problematic lesions (such as CPDs), whereas the more complex error-free template switching might be necessary for lesions which are resistant to TLS.

Conclusion Remarks and Prospects

Because of the DNA damage bypass pathways, genomic replication can be completed in the presence of DNA damage. Recent studies have highlighted that these pathways are mechanistically separable from genome replication and are able to function after the bulk of replication is completed. However, how genome replication is enabled in the presence of DNA lesions remains poorly understood. Repriming events downstream of lesions and additional origin firing are proposed to assist the efficiency of replication when replication forks are arrested by such lesions. In budding yeast, the replication origins are strictly defined by the consensus sequence and therefore repriming events are potentially more used for the resumption of replication downstream of a lesion. In other eukaryotic sys-

tems, including fission yeast, fruit fly and human cells, replication origins are less defined and this initiation may be endowed with additional flexibility in terms of location, implying that the arrested replication fork could be more often met by another fork initiated within the unreplicated region. However, under this hypothesis, lesions would need to be more widely spaced compared to replication initiation sites in order to avoid problems.

With regard to the repriming events, their existence needs to be confirmed in eukaryotes and any potential mechanism remains to be characterized. Analysis in *E. coli* has shown that the replisome is able to reprime leading strand synthesis downstream of the lesion through various mechanisms. Many of the mechanistic principals of replisome components, such as the replicative helicase, polymerase and sliding clamp are well conserved between prokaryotes and eukaryotes. However, there are important differences. First, conserved factors for reloading replisomes have not been identified in eukaryotes. Indeed, they may not exist since the replication machinery is tightly regulated to avoid re-replication and is also protected by checkpoint functions. In contrast to bacterial systems, replication from neighbouring origins can compensate for arrested and broken replication forks. Second, eukaryotic cells have Polδ and Polε as replicative polymerases while PolIII is the only replicative polymerase in *E. coli*. In budding yeast and fission yeast, Polδ is reported to function for lagging strand DNA synthesis while Polε replicates the leading strand (97,98). It could be suggested that, in the presence of DNA lesions, DNA synthesis involving Polα/primase and Polδ takes part in repriming and subsequent extension of leading strand synthesis, which would be reminiscent of repriming on the lagging strand. Such a possibility would be consistent with the fact that the catalytic activity of Polε is not essential for viability of budding yeast and fission yeast (99,100); potentially, Polδ is able to substitute for the role of Polε.

The members of the *RAD6* epistasis group have been the main target of characterization for the elucidation of DNA damage bypass mechanisms. However, the involvement of other factors that have essential cellular functions, for example in genome replication, maintenance of chromosomes etc., could potentially have been underestimated because of the limited availability of mutants. Indeed, studies with hypomorphic mutants of Polδ showed its involvement in PRR (101). Intriguingly, human Polη is localized at replication factories and appears to be functional in unperturbed cells (102–104). Taken together, it can be postulated that DNA lesion-bypassing mechanisms are more tightly related to and coupled with the replication machinery than has been appreciated to date.

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