

Error-free Recombinational Repair Predominates over Mutagenic Translesion Replication in *E. coli*

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Summary

Tolerance mechanisms are important in the ability of cells to cope with DNA damage. In *E. coli*, the two main damage tolerance mechanisms are recombinational repair (RR) and translesion replication (TLR). Here we show that RR effectively repairs gaps opposite DNA lesions. When both mechanisms are functional, RR predominates over TLR, being responsible for 86% of the repair events. This predominance of RR is determined by the high concentration of RecA present under SOS conditions, which causes a differential inhibition of TLR. Further inhibition of TLR is caused by the RecA-catalyzed strand exchange reaction of RR. This molecular hierarchy in the tolerance of DNA lesions ensures that the nonmutagenic RR predominates over the mutagenic TLR, thereby contributing to genetic stability.

Introduction

Cellular responses to DNA damage function to ensure genomic stability. In *E. coli*, as in other organisms, there are several pathways of DNA repair which operate with a certain hierarchy to minimize the deleterious effects caused by damage inflicted on the genome (Friedberg et al., 1995). Most lesions are eliminated from DNA by error-free repair mechanisms, primarily excision repair. However, some lesions escape repair and cause replication blocks, leading to the formation of single-stranded gaps opposite the lesions. Such ssDNA regions are prone to breakage and must be filled in for the cell to survive (Friedberg et al., 1995; Livneh et al., 1993). At least two mechanisms can fill-in such gaps: recombinational repair involves patching of the gap by a segment of complementary DNA taken from the sister chromatid strand (Cox, 1999; Eggleston and West, 1996; Kowalczykowski et al., 1994). This process is inherently nonmutagenic. Once the gap is filled, the lesion is present in a duplex region again, and a second attempt of error-free excision repair can be made. While this mechanism was suggested long ago (Rupp and Howard-Flanders, 1968; Rupp et al., 1971), there has been little direct evidence for its existence (reviewed in Courcelle et al., 2001). A second, more established gap-filling mechanism is TLR, in which the gap is filled-in by DNA synthesis, catalyzed by DNA polymerase V (Goodman, 2000; Livneh, 2001). Pol V, which belongs to the Y family of DNA polymerases (Ohmori et al., 2001), specializes in replicating across

DNA lesions (Reuven et al., 1999; Tang et al., 1999), a reaction which is inherently mutagenic, due to the miscoding nature of most DNA lesions. In addition, pol V is an error-prone polymerase even when acting on undamaged regions of DNA (Maor-Shoshani et al., 2000; Tang et al., 2000). It was suggested that TLR is the last recovery mechanism to act after DNA damage, rationalized by the argument that the cell resorts to mutagenic repair only as a last option (Sommer et al., 1993). Moreover, it was suggested that once induced, pol V and UmuD' inhibit RR, therefore switching the mode of the cell to error-prone repair (Sommer et al., 1993).

The balance between error-free RR and mutagenic TLR determines the final mutagenicity of unrepaired DNA lesions. Therefore, the relationship between the two is of considerable interest. Here we present direct evidence that RR effectively fills-in gap-lesion structures, and show that there is a built-in hierarchy in DNA damage-tolerance mechanisms, which favors the non-mutagenic RR over mutagenic TLR.

Results

An Assay System for Gap Filling by Recombinational Repair

The gap-filling system (Figure 1) is based on a gapped plasmid, termed GP21, which carries a synthetic abasic site in a single-stranded region of 22 nucleotides, and a kanamycin resistance gene (Tomer and Livneh, 1999). The ability of GP21 to survive in an *E. coli* host depends on filling-in of the gap, and was used as a measure for gap filling. The survival of plasmid GP21 was assayed by the number of kan^r colonies obtained when it was introduced into an *E. coli* host, normalized to the number of kan^r colonies obtained with a control gapped-plasmid termed GP20, which is similar to GP21 except that it does not contain a lesion (Reuven et al., 1998). When no homologous DNA is present and RR is impossible, the gap can be filled in by pol V-dependent TLR (Reuven et al., 1998). The same situation exists when pUC18, a heterologous partner plasmid is present (Figure 1). However, when a homologous plasmid is present in the cell (FGP20/*Tamp*), the gap in GP21 can be filled in, theoretically, either by TLR or by RR (Figure 1).

Filling-in the gap by recombination implies that a marker present on the homologous region of the partner plasmid must be transferred to the gapped plasmid during repair. The homologous partner plasmid was chosen to contain a T at the position corresponding to the base opposite the lesion in GP21. Therefore, if the gap is filled in by recombination, a T is expected at that site in the recovered GP21 plasmids. This facilitates discrimination between gap filling by recombination, or TLR, since pol V-dependent TLR, which is the main TLR reaction, leads to the insertion of primarily an A opposite the abasic site (T is infrequently inserted, <3%) (Reuven et al., 1998, 1999), whereas bypass by pol III holoenzyme creates primarily minus 1 deletions (Reuven et al., 1998; Tomer et al., 1998).

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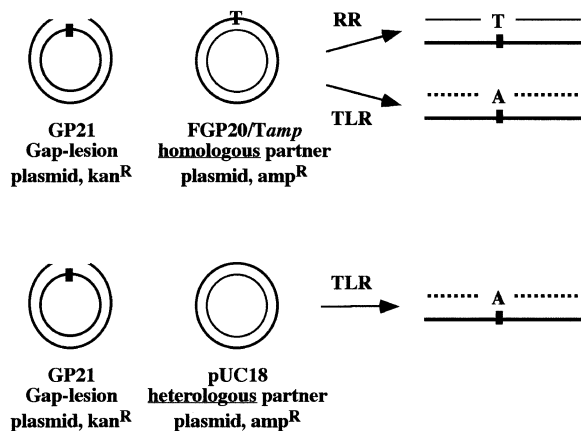


Figure 1. Outline of the In Vivo Assay System for Gap Filling by Recombinational Repair

The gap-lesion plasmid GP21 (kan^R) is introduced into *E. coli* cells along with a homologous partner plasmid (FGP20/Tamp; amp^R). Parallel experiments are carried out with a heterologous plasmid (pUC18). Only gap-filling repair will allow GP21 to transform the cells and confer a kan^R phenotype. This can be done by TLR, which does not depend on the partner plasmid, or by RR, which depends on a homologous partner plasmid. The homologous partner plasmid carries a T opposite the site corresponding to the lesion in GP21, so GP21 filled in by recombination will have a T at that position. In contrast, GP21 filled-in by pol V-dependent TLR will have primarily an A at that location (Reuven et al., 1998).

Gap-Lesion Structures Are Efficiently Repaired by Recombinational Repair

Gap filling by RR was assayed by the ability of plasmid GP21, in the presence of a homologous partner plasmid, to provide *E. coli* cells with kanamycin resistance. The cells were not treated for SOS induction, and therefore gap filling via TLR could not occur (Reuven et al., 1998). First, a sequential transformation protocol was adopted. *E. coli* cells were transformed with the homologous partner plasmid (amp^R). After establishment of transformants, the gap-lesion plasmid was introduced by a second transformation, and cells were plated in the presence of kanamycin. As can be seen in Table 1A, in the absence of the homologous partner plasmid, survival of the gap-lesion plasmid in noninduced cells was very low (1.1%). When a homologous partner plasmid was present, survival increased dramatically by 46-fold,

up to 51% (Table 1A), suggesting the involvement of a recombinational repair process. In this experimental protocol, the incoming gap-lesion plasmid encounters multiple copies of the resident homologous plasmid. Similar results were obtained by using another protocol in which the two plasmids were cotransfected at the same time. The survival of the gap-lesion plasmid was very low when cotransfected with a heterologous plasmid (1.4%), but when cotransfected with the homologous plasmid, survival increased 18-fold, reaching 27% (Table 1A). These results suggest that the partner plasmid provided homologous DNA sequences, which were used in the process of filling-in the gap.

The homology-dependent repair of the gap-lesion plasmid was examined in several genetic backgrounds. As can be seen in Table 1C, the recovery was dependent on the RecA recombinase, but not on the *umuDC* operon, encoding the lesion bypass-specific DNA polymerase V. Derepression of the SOS regulon by a *lexA51* mutation did not alleviate the requirement for RecA (Table 1C). Thus, the repair of GP21 requires the RecA recombinase and a homologous plasmid, but not pol V. These properties are characteristic of a recombination reaction.

Table 2A shows the nucleotide present at the location corresponding to the site opposite the original abasic site in repaired GP21 plasmids recovered from noninduced *E. coli* cells. As can be seen, a total of 46 out of 49 (94%) plasmids had a T at that site, indicating an RR reaction. Of these, 17/18 were obtained in a protocol of two consecutive transformations, and 29/31 were obtained in a cotransformation procedure (Table 2A). In control experiments using a heterologous plasmid, 36/36 (100%) of the recovered plasmids carried a minus 1 deletion (Table 2A), typical of pol III holoenzyme action (Reuven et al., 1998; Tomer et al., 1998). Taken together these results indicate that in this system gap-lesion structures are repaired via recombinational repair.

Recombinational Repair Predominates over TLR In Vivo

In SOS-induced *E. coli* cells harboring both plasmid GP21 and the homologous partner plasmid, the gap-lesion can be filled-in either by recombination or by TLR. This gave us the opportunity to determine which of the two gap-filling mechanisms dominates in this system.

Table 1. Repair of a Gap-Lesion Plasmid Requiring a Homologous Intact Partner Plasmid

Genotype, Induction	Homologous plasmid	Plasmid Survival, %			
		Sequential Transformation		Cotransformation	
		-	+	-	+
A Wild-type, noninduced		1.1	51	1.4	27
B Wild-type, SOS-induced		5.5	71	4.4	25
C $\Delta recA$, noninduced				1.9	2.6
$\Delta recA lexA51$, SOS-induced				1.5	2.9
$\Delta umuDC$, SOS-induced				1.3	30

The gap-lesion plasmid GP21 was introduced into SOS-induced or noninduced *E. coli* AB1157 cells, as indicated, along with a homologous intact plasmid (FGP20/Tamp) or a heterologous intact plasmid (pUC18; cotransformation). Alternatively, the partner plasmid was introduced first, and transformed cells were subjected to a second transformation with the gap-lesion plasmid (sequential transformation). Plasmid survival was calculated by dividing the number of transformants by that obtained with GP20, the control plasmid without a lesion. The strains used in (C) were WBN1 $\Delta recA$, WBJ11 $\Delta recA lexA51$, and WBY100 $\Delta umuDC$.

Table 2. Specificity of the Nucleotide Inserted Opposite the Lesion during Gap-Filling Repair

Homologous plasmid	Nucleotide Inserted Opposite the Lesion			
	Sequential Transformation		Cotransformation	
	-	+	-	+
A Noninduced Cells				
T	1	17	0	29
A	0	0	0	0
G	0	0	0	0
C	0	0	0	0
-1	23	1	36	2
Total	24	18	36	31
B SOS-Induced Cells				
T	2	69	0	59
A	26	1	19	7
G	3	0	2	1
C	0	0	1	0
-1	16	1	15	2
Total	47	71	37	69

The experiments were performed as described in the legend to Table 1, with *E. coli* AB1157. Plasmids were extracted from individual colonies and used to transform a *recA* strain to *kan^R*. Repaired GP21 plasmids were isolated from these secondary transformants and subjected to DNA sequence analysis.

Table 1B summarizes the results obtained in SOS-induced cells, when both TLR and RR can occur. When assayed in the presence of a heterologous plasmid, survival of GP21 was 3-5 fold higher in UV-irradiated cells, as compared to unirradiated cells, reaching up to 5.5%. This is consistent with previous results (Henderson et al., 2002; Lawrence et al., 1990; Reuven et al., 1998) and is attributed to TLR by pol V (Reuven et al., 1998). When assayed in the presence of the homologous partner plasmid, survival was much higher than with the heterologous plasmid. In the sequential transformation procedure, survival reached 71%, whereas in the cotransformation procedure, it reached 25% (Table 1B). These results indicate that RR is much more effective than TLR in filling-in the gap containing the lesion. Notice that there was little effect of SOS induction on the efficiency of RR in this system.

DNA sequence analysis was performed on plasmids recovered from SOS-induced cells to determine the identity of nucleotide present opposite the site of the original abasic site. As can be seen in Table 2B, in the presence of the heterologous plasmid, about half of the plasmids contained an A at that site (a total of 45/84; 54%), consistent with the activity of pol V-promoted TLR, as previously reported (Lawrence et al., 1990; Reuven et al., 1998). Most other plasmids contained (-1) deletions, characteristic of TLR by pol III holoenzyme (Reuven et al., 1998; Tomer et al., 1998). In contrast, when repair occurred in the presence of the homologous plasmid, an overwhelming majority of plasmids carried a T at that site, characteristic of RR. In the sequential transformation, 69/71 (97%) plasmids contained a T, and in the cotransformation, 59/69 (86%) of the plasmids carried a T. Most of the other plasmids contained an A, indicating TLR by pol V. Taken together these results indicate

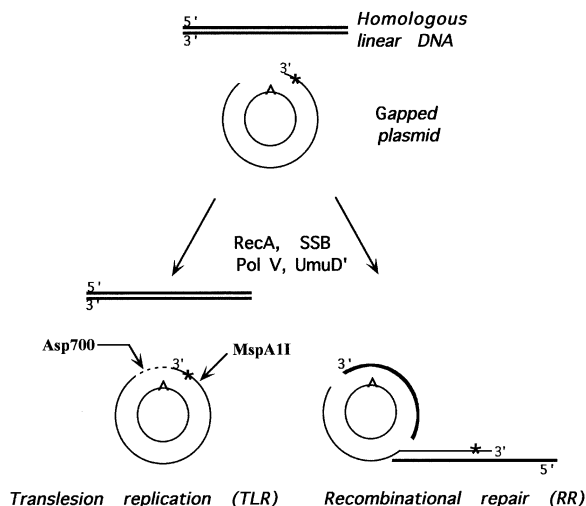


Figure 2. Schematic Presentation of the Gap-Filling Reactions in the Simultaneous RR and TLR Assay System

Upon addition of RecA, SSB, pol V, UmuD', and homologous linear (hl)-DNA, the single-stranded gap opposite the lesion can be either filled by pol V-dependent TLR, or patched by the homologous DNA via a strand exchange recombination reaction (RR). The restriction sites indicated are used in the analysis of TLR, whereas the RR product is assayed by its slow electrophoretic mobility. See text for details.

that in SOS-induced cells, where both TLR and RR can occur, RR dominates over TLR and is responsible for at least 86% of the repair events.

Recombinational Repair Suppresses Translesion Replication in an In Vitro Model System

In order to gain insight into the mechanisms underlying the dominance of RR over TLR, we used an in vitro model system, in which both mechanisms could operate simultaneously. The details of the system will be described elsewhere. Briefly, the system consists of plasmid GP21, which was used in the in vivo studies described above, except that it contains a radiolabeled internal phosphate, 15 nucleotides upstream to the 3' terminus of the primer strand, and a larger gap of approximately 350 nucleotides (Figure 2). In addition, the reaction mixture contains a partner linear dsDNA (1085 bp), homologous to the gap-lesion plasmid, with one end corresponding to the gap region, approximately 200 nucleotides 5' to the lesion (Figure 2). When pol V, UmuD', RecA, and SSB are added to these two substrates, in the presence of ATP, Mg²⁺, and dNTPs, two reactions can occur. (a) Pol V promoted TLR, as previously described (Reuven et al., 1999). (b) RecA promoted strand exchange, leading to the formation of a joint molecule composed of the two DNA substrates (Figure 2) (A.B and Z.L., unpublished data). This strand exchange reaction serves as a simplified model system for RR. It should be pointed out that the optimal conditions for the TLR reaction were similar to those of the model RR reaction so that both reactions could be performed simultaneously without special adjustment. In order to follow each of the reactions separately, after completion of the reaction, the samples were divided

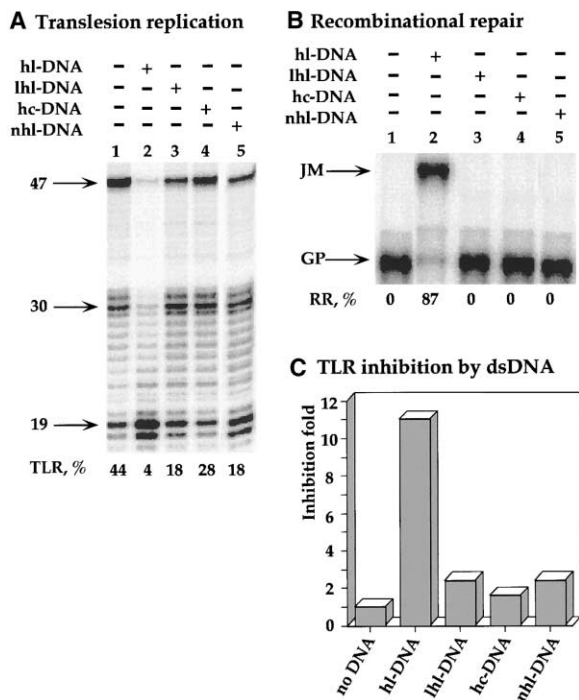


Figure 3. A Homologous Partner DNA with a Recombinogenic End Inhibits TLR in the RR/TLR Assay System

Simultaneous TLR and RR reactions were performed for 30 min, as described in Experimental Procedures, either without a partner DNA (lane 1), or with one of the following DNA molecules: homologous linear DNA (hl-DNA, lane 2), long homologous linear DNA (lhl-DNA, lane 3), homologous circular DNA (hc-DNA, lane 4), or nonhomologous linear DNA (nhl-DNA, lane 5). The TLR products are shown in (A), and the RR products are shown in (B). The extent of TLR inhibition caused by addition of various DNA substrates is shown in (C).

into two aliquotes and assayed separately for TLR and RR. TLR was followed as previously described by cutting the filled GP21 with two restriction nucleases and analyzing the synthesis products by urea-PAGE (Reuven et al., 1999). RR was assayed by fractionating the reaction products by agarose gel electrophoresis, followed by phosphorimaging.

Figure 3 shows the results of TLR and RR occurring simultaneously in the same test tube, and assayed in parallel for TLR (Figure 3A) and for recombination (Figure 3B). The extent of TLR was determined by the percentage of radiolabeled products which have been extended past the abasic site (31–47 nucleotides long, Figure 3A), out of all primers. The extent of RR is presented by the percentage of GP21 converted to joint molecules (JM, Figure 3B). In the absence of the homologous linear DNA, where the model RR reaction could not occur, TLR reached 44% (Figure 3A, lane 1). In the presence of hl-DNA, TLR was drastically reduced 11-fold, to 4% (Figure 3A, lane 2). Under the same conditions, RR was highly effective, with 87% of the gapped plasmids converted to joint molecules (Figure 3B, lane 2). Similar reactions were conducted using three other types of partner DNAs: a nonhomologous DNA (nhl-DNA), a homologous circular DNA (i.e., with no ends; hcDNA), and a longer homologous linear DNA (lhl-DNA) whose ends correspond to double-stranded regions of the plasmid flank-

ing the gap. Thus, the recombinogenic 3' end of the lhl-DNA substrate is homologous to the double-stranded region of the gapped plasmid and not to its ssDNA region. None of these DNA substrates was able to support a strand exchange recombination reaction with the gapped plasmid (Figure 3B, lanes 3–5). As can be seen in Figure 3A, all these DNAs caused only a minor inhibition of TLR 1.6- to 2.4-fold (Figures 3A and 3C). Thus, RR suppressed TLR in this in vitro system, and this suppression was dependent both on homology, and on the presence, in the partner DNA, of a 3' end corresponding to the ssDNA region. This indicates that it is not merely the presence of the partner DNA that inhibits TLR, but also an active recombination reaction is required for inhibition.

High Concentrations of pol V and UmuD' Inhibit Strand Exchange

Were the recombination reaction and TLR competing for the same substrate (i.e., the gap-lesion), then increasing the concentration of pol V is expected to favor TLR over recombination. To test this possibility, recombination was assayed with increasing concentrations of pol V, or pol V and UmuD'. Figure 4A shows that addition of pol V at concentrations of 1 or 1.5 μ M, in the presence UmuD', led to a significant inhibition of strand exchange of 13- and 15-fold, respectively (Figure 4A, lanes 8 and 9). Pol V alone also inhibited joint molecule formation, although to a lesser extent of 5- and 6-fold, respectively (Figure 4A, lanes 5 and 6). Inhibition of recombination by pol V and UmuD' occurred both in the presence or absence of SSB. Addition of UmuD' alone did not affect recombination (data not shown). Kinetics of recombination, with or without pol V and UmuD', showed that the inhibitory effect of the Umu proteins occurs early on during the recombination reaction (Figure 4B). A similar effect was observed with a gapped plasmid without a lesion (data not shown). These results are consistent with a previous study reporting that pol V, as a UmuD'₂C complex, binds in a stoichiometric fashion along RecA-ssDNA nucleoprotein filaments, thereby inhibiting RecA-catalyzed strand exchange (Rehrauer et al., 1998). The fact that a comparable concentration-dependent inhibition of strand exchange was obtained with the MBP-pol V protein and with the UmuD'₂C complex of pol V suggests that the MBP domain does not significantly interfere with the ability of pol V to compete against RecA-mediated strand exchange.

Increased RecA Concentration Alleviated pol V-Mediated Inhibition of Strand Exchange Repair, and Inhibited Translesion Replication

The opposing effects that RecA and pol V have on recombination suggested that the eventual outcome will depend on their relative concentrations. Under SOS conditions, the average in vivo concentrations of pol V, UmuD', and RecA were estimated to be 0.3, 3.6 (Woodgate and Ennis, 1991), and 90 μ M (Salles and Paoletti, 1983), respectively. This means that RecA is present at a 300-fold excess over pol V. We therefore examined the effect of increasing RecA concentration in the presence of pol V at concentrations that inhibited the recombination reaction. Figure 5 shows the result of an experi-

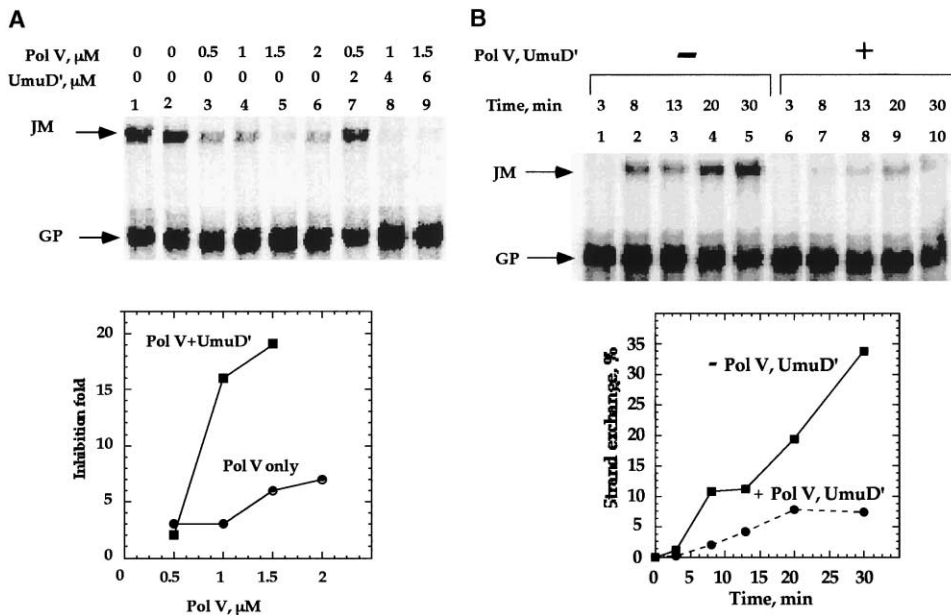


Figure 4. High Concentrations of pol V and UmuD' Inhibit RR

(A) Effect of pol V and UmuD' concentrations on RR. RR reactions were performed as described in Experimental Procedures, with 2 μM RecA and 50 nM SSB and the indicated concentrations of pol V (lanes 3–6), or pol V and UmuD' (lanes 7–9). Lanes 1 and 2 contained control reactions without pol V and UmuD'.

(B) Time course of strand exchange in the presence of inhibitory concentrations of pol V and UmuD'. RR reactions were performed for the indicated time periods with 2 μM RecA and 50 nM SSB, with (lanes 6–10) or without (lanes 1–5) 1.5 μM pol V and 6 μM UmuD'. The graphs show the quantification of the phosphorimages.

ment in which recombination and TLR were assayed in the presence of 1.5 μM pol V, 6 μM UmuD', and 4–32 μM RecA. When only one type of reaction was allowed, TLR reached 70% (Figure 5A, lane 1), and recombination reached 80% (Figure 5B, lane 1). Under conditions where the two reactions occurred simultaneously, and RecA was kept at a low concentration (4 μM), 43% of the molecules were bypassed by TLR (Figure 5A, lane 2), whereas only 31% were bypassed by recombination (Figure 5B, lane 2). When RecA concentration was increased to 16 or 32 μM , recombination was as high as in the absence of pol V, whereas TLR was strongly suppressed. Thus, at 32 μM RecA, 82% of the molecules were bypassed by recombination (Figures 5B, lane 6, and 5C, column 9), whereas only 6% were bypassed by TLR (Figures 5A, lane 6, and 5C, column 9). We have not increased RecA concentrations above 32 μM due to technical difficulties. However, it is clear that under protein concentrations relevant to the in vivo situation, where RecA is in high excess over pol V, recombination dominated over TLR, fully consistent with our in vivo results. This dominance of recombination was achieved by two mechanisms: (a) high concentrations of RecA inhibited TLR (Figures 5A, lanes 1, 3, and 5; and 5C, columns 1, 4, and 7), while fully promoting recombination (Figures 5B, lanes 1, 3, and 5; and 5C, columns 2, 5, and 8); and (b) recombination inhibited TLR (Figures 5A, lanes 2, 4 and 6; and 5C, columns 3, 6, and 9).

Discussion

The term recombinational repair is often used to describe different pathways of recovery from DNA damage

(Cox, 1999; Kowalczykowski et al., 1994). The precise molecular mechanisms of these pathways are only partially understood, due to the scarcity of well-defined assays for distinct recombination reactions. As a result, it is often difficult to interpret in vivo data on recombinational repair in molecular terms (Courcelle et al., 2001). RecA and recombinational mechanisms have at least two different and distinct roles in the recovery of *E. coli* from UV light DNA damage: (a) resumption of DNA replication, which involves reestablishment of replication forks, involves a recombinational reaction, which is RecA dependent, but does not necessarily involve reciprocal strand exchange (Kowalczykowski, 2000) (this is a major survival factor for *E. coli*, since its malfunction endangers the entire chromosome [Courcelle et al., 2001]); and (b) filling-in of replication gaps caused by unrepaired lesions. Although it has been proposed long ago that this recombination reaction is an important repair mechanism (Rupp and Howard-Flanders, 1968; Rupp et al., 1971), there was little experimental data to support it, as pointed out recently (Courcelle et al., 2001).

Our results provide direct evidence for DNA gap filling by RecA-promoted recombinational repair. This type of repair occurs rather effectively in our in vivo system, reaching up to 65% gap filling. Like any other bimolecular reaction, the recombination reaction is expected to be concentration dependent. This can explain the fact that RR was higher (51%) when the incoming gap-lesion plasmid encountered multiple copies of the partner plasmid (consecutive transformation protocol) compared to equal initial number of copies/cell of both partner and gap-lesion plasmids (25%), as in the cotransformation protocol. In the *E. coli* chromosome, gap-filling RR is

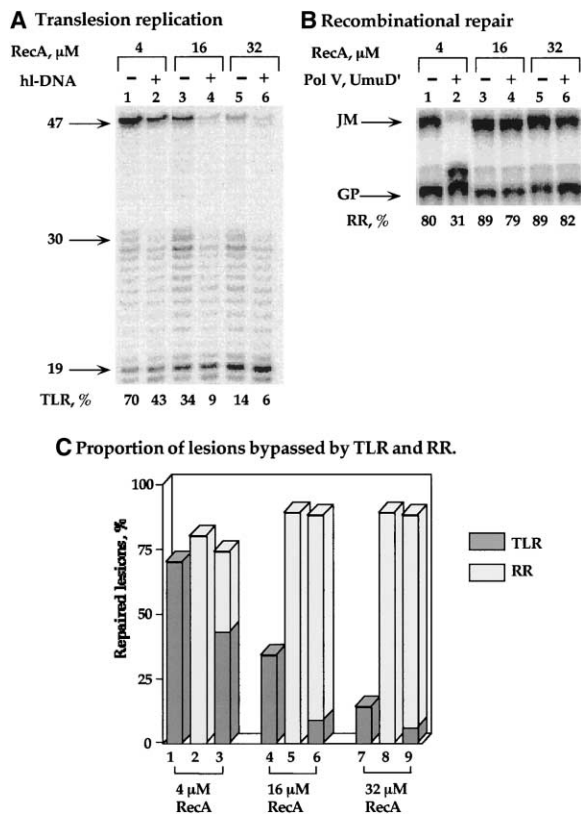


Figure 5. Increased RecA Concentrations Alleviate UmuD' C-Mediated Inhibition of RR and Inhibit TLR

Simultaneous reactions were performed as described under Experimental Procedures, with 1.5 μ M pol V, 6 μ M UmuD', 200 nM SSB, and the indicated RecA concentrations. (A) shows the TLR products in the simultaneous reactions (lanes 2, 4, and 6) and in the TLR-only control reactions (lanes 1, 3, and 5). (B) shows RR products in the simultaneous reactions (lanes 2, 4, and 6) and RR only reactions (lanes 1, 3, and 5). (C) shows the percentage of lesions tolerated by TLR (dark gray) and RR (light gray) in the TLR-only reactions (columns 1, 4, and 7), RR-only reactions (columns 2, 5, and 8), and RR and TLR occurring simultaneously (columns 3, 6, and 9). The data was taken from (A) and (B).

expected to occur between two sister chromatids, which are present in great proximity. This means that the recombining DNAs are at high local concentrations, suggesting that the process might be even more efficient than in our plasmid system.

Our results show that there is a built-in hierarchy in gap-filling repair, with gap-filling RR dominating over TLR. Based on our in vitro results, this dominance is achieved by two mechanisms: (a) a differential effect of high RecA concentration—it inhibited TLR while effectively promoting RR; and (b) the recombination reaction inhibits TLR. The finding that RecA causes inhibition of TLR was somewhat surprising in light of the fact that RecA (Reuven et al., 1998; Tang et al., 1998), and in particular its assembly as a RecA nucleoprotein filament, is required for TLR (Reuven et al., 2001). We have previously reported that the actual binding of pol V to the primer terminus, and its polymerization activity, required dissociation of RecA from DNA (Reuven et al., 2001). Similarly, RecA dissociated during the elongation stage

of TLR (Pham et al., 2001). This suggests that the RecA filament has a dual effect on TLR: it is essential for targeting pol V to the DNA, but once pol V is on the DNA, RecA becomes an obstacle to TLR and has to dissociate. Higher RecA concentrations stabilize the RecA nucleoprotein filament and are therefore inhibitory to TLR. As for the second mode of inhibition, our in vitro results showed that inhibition of TLR by the partner DNA was observed only when the latter was homologous to the gapped plasmid and capable of performing the recombination reaction. This indicates that steps such as RecA-promoted homology search do not cause a strong inhibition of TLR. Rather, the inhibition requires the formation of a productive strand exchange ternary complex between RecA, the gapped plasmid, and the linear duplex, which catalyzes strand transfer and strand exchange. Inhibition is caused, most likely, due to a steric hindrance to the activity of pol V.

The dominance of gap-filling RR over TLR makes sense from the standpoint of genomic stability, since RR is nonmutagenic whereas TLR is fundamentally mutagenic (Livneh, 1993, 2001; Radman, 1999). What does the dominance of RR teach us about the function of TLR? One possibility is that TLR deals with a subpopulation of gap-lesion structures, which cannot be tolerated by RR. Such cases can occur when there is no intact homologous donor DNA, for example, at overlapping daughter strand gaps or after an excision repair attempt at closely opposing lesions. Under such circumstances, TLR appears to be the only known mechanism for filling-in the gap-lesions in DNA. In addition, TLR can serve as a low-priority backup mechanism for RR. It is usually strongly suppressed, even when SOS is induced, to keep a tight control over the rate of mutation formation. Based on in vivo experiments presented above, and on other studies (Banerjee et al., 1988; Lawrence et al., 1990; Reuven et al., 1998), TLR across blocking lesions is an inefficient process (bypass of abasic sites was approximately 5%). This is not the result of a built-in problem in the bypass ability of pol V, since TLR can reach very high efficiencies of up to 70% in vitro, as shown above. Rather, it is likely to represent tight regulation over a process that is inherently mutagenic. This is consistent with our finding that high RecA concentrations inhibit TLR, even in the absence of recombination. These efficiencies might be even lower for lesions in the chromosome, where RR is operational. It was suggested that when *E. coli* cells are exposed to harsh environmental condition, pol V and pol IV function as mutases to increase mutation rates, thereby facilitating adaptation to the environment (Echols, 1981; Radman, 1999; Witkin and Wermundsen, 1979). This notion is consistent with our results, which add a new layer of regulation on the process of induced mutagenesis in order to prevent uncontrolled accumulation of mutations.

It was previously reported that overproduction of UmuD' and pol V caused a reduction in recombination and UV survival, and an increase in UV mutagenesis (which occurs by pol V-dependent TLR). Increasing RecA concentrations caused a decrease in UV mutagenesis (Boudsocq et al., 1997). Although these results were interpreted as suggesting that the induction of UmuD' C shuts off RR, our results clearly show that when SOS and UmuD' C are induced, RR still dominates over TLR.

The inhibitory effect of umuD' C on RR in that study was seen only under artificial overproduction of UmuD' C and does not represent a physiological situation. However, these results do indicate that the two reactions compete for the same DNA substrate in vivo, consistent with our in vivo and in vitro results.

In summary, we have shown that (1) RR effectively repairs gap-lesion structures in vivo, and (2) there is a built-in hierarchy that favors the nonmutagenic RR over mutagenic TLR. This dominance of RR is determined by the high concentration of RecA under SOS conditions and is achieved by two mechanisms: (a) a differential effect of RecA, which at high concentrations inhibits TLR while fully promoting RR; and (b) the strand exchange reaction itself, promoted by RecA, inhibits TLR.

Experimental Procedures

Materials

The sources of SSB, RecA, MBP-pol V, and UmuD' were previously described (Reuven et al., 1998). Radiolabeled nucleotides were from NEN research products; Restriction nucleases, T4 DNA ligase and T4 polynucleotide kinase were from New England Biolabs. T7 gp6 exonuclease was from Amersham. dNTPs, Proteinase K, and Asp700 were from Boehringer-Mannheim.

Bacterial Strains

The bacterial strains used in this work are *E. coli* JM109 *thi rpsL(Str^h) endA sbcB15 sbcC hsdR4(r_k⁻m_k⁺) Δ(lac-proAB) recA1 F' traD36 lacI^o Δ(lacZ)M15 proA⁺B⁺*, and derivatives of *E. coli* AB1157 *argE3 hisG4 leuB6 proA2 thr1 ara14 galk2 lacY1 mtl1 xyl5 thi1 tsx33 rpsL31 supE44* as follows: ZTR10, same as AB1157 but also *tna::Tn10*; WBN2, same as AB1157 but also *ΔrecA306::Tn10*; WBJ11, same as AB1157 but also *sfiA11 lexA51 ΔrecA306::Tn10*; and WBY100, same as AB1157 but also *ΔumuDC*.

DNA

All oligonucleotides were synthesized and purified by the Synthesis Unit of the Biological Services Department of the Weizmann Institute of Science. Oligonucleotides containing an abasic site analog were synthesized similarly using dSpacer CE phosphoramidite as a building block (Glen Research, Sterling VA). The gap-lesion plasmids GP21 and GP20 were constructed by ligating a gapped duplex oligonucleotide insert carrying a site-specific abasic site analog to a restriction nuclease-cleaved plasmid via nonpalindromic cohesive termini, as previously described (Reuven et al., 1998). Plasmid FGP20/T-amp is an ampicillin-resistant kanamycin-sensitive derivative of GP20 that is fully double stranded. FGP20/T-amp contains a T opposite the location corresponding to the abasic site, and it has complete homology to the abasic site flanking regions in GP21 (Avkin et al., 2002).

Homologous circular DNA (hc-DNA) was prepared by transformation of gapped plasmid that did not contain an abasic site into *E. coli* JM 109. Then the plasmid (filled in *E. coli*) was purified by Qiagen maxi-prep kit. The homologous linear DNA (hl-DNA) was obtained by digestion of hc-DNA with restriction endonucleases Dra I and Hind III, fractionation on a 0.8% agarose gel, and electroelution of the DNA fragment (1085 bp). Long homologous linear DNA (lhl-DNA) was prepared similarly by digestion of hc-DNA plasmid with AlwN I and Hind III endonucleases, producing a 1450 bp fragment. Heterologous linear DNA substrate (nhl-DNA) was prepared from pMAC plasmid, digested with Dra I and Sma I endonucleases, and the 1095 bp fragment was obtained by gel fractionation and electroelution.

In Vivo RR and TLR Assay

The assay involves cotransformation of the UV-irradiated *E. coli* cells with a mixture of the gap-lesion plasmid (GP21; kan^r) and the homologous partner plasmid (FGP20/T-amp). In parallel, as a control, the cells are transformed with a mixture of the gapped plasmid without a lesion (GP20; kan^r) and the homologous partner plasmid. Irradiated cells were grown on LB medium to O.D.₅₉₅ 0.5,

after which they were concentrated by centrifugation and transferred to 10 mM Tris-Cl, 150 mM NaCl. The cells were then UV irradiated (254 nm) with 20 J/m², diluted by the addition of an equal volume of 2-fold concentrated LB medium, and incubated for 30–60 min at 37°C for expression of SOS functions. The induced cells were transformed with a mixture containing either GP21 or GP20 (100 ng), along with the homologous plasmid FGP20/T-amp, or the heterologous plasmid pUC18 (100 ng). The cells were plated on LB plates containing ampicillin (100 mg/l) and kanamycin (50 mg/l). The extent of gap filling was deduced from the number of kan^r colonies obtained with the GP21-containing mixture, normalized to the number of transformants obtained with the GP20-containing mixture. Each experiment was performed two to three times, in duplicates, and in some cases, in triplicates. The variation among experiments was in the range of 20%–25%. In order to determine the DNA sequence at the site where the lesion was located, plasmids were isolated from the transformed cells and fractionated by electrophoresis on low-melting agarose. A gel slice containing the filled-in GP21 was melted and used to transform a fresh culture of *E. coli*. Repaired GP21 plasmids were isolated from kanamycin-resistant, ampicillin-sensitive colonies of this secondary transformation and subjected to DNA sequence analysis. The mutants analyzed from each type of experimental conditions were taken from at least four independent experiments.

Combined Model Assay System for Translesion Replication and Recombinational Repair

The typical combined TLR and RR reaction mixture (50 μl) contained 20 mM Tris-HCl, pH 7.5, 8 μg/ml bovine serum albumin, 5 mM DTT, 0.1 mM EDTA, 4% glycerol, 2.5 mM ATP, 10 mM MgCl₂, 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dTTP, 0.1 mM dCTP, 0.2 μg/ml creatine kinase, 10 mM creatine phosphate, 0.2 μg (2 nM) gapped plasmid, 50 nM SSB tetramers, 4 μM RecA, 400 nM UmuD', 100 nM MBP-pol V, and 0.3 μg (8 nM) of hl-DNA. The combined reactions were started by simultaneous addition of pol V, UmuD', and hl-DNA. Reactions were carried out at 37°C for the indicated periods of time and then divided to two equal parts: one for the analysis of TLR and another for the analysis of RR. Analysis of RR was performed by fractionating the reaction mixture by electrophoresis on a 0.8% agarose gels, at 45V for 16 hr. Then, the gels were dried, and visualized and quantified using a Fuji BAS 2500 phosphorimager. The extent of strand exchange was calculated by dividing the amount of joint molecules produced by the amount of all labeled DNA in the reaction: the sum of joint molecules and gapped plasmid. Analysis of TLR was performed as previously described. Briefly, reactions were terminated by adding SDS to 0.2%, EDTA to 20 mM, and NaCl to 100 mM, heat inactivated, and digested with proteinase K, after which the DNA was extracted with phenol-chloroform and was ethanol precipitated. The DNA was digested with restriction nucleases Asp700 and MspA11 in order to reduce the size of replication products and thereby increase resolution. This produced radiolabeled DNA fragment of 19 nt in the case the primer was not extended, 29 or 30 nt fragment when the polymerization was stopped by the lesion, and 47 nt fragment in the case of full bypass product, restricted with downstream Asp700 cleavage. The DNA samples were fractionated by electrophoresis on 15% polyacrylamide gels containing 8 M urea, dried, and visualized and quantified using a Fuji BAS 2500 phosphorimager. The extent of TLR was calculated by dividing the amount of bypass products by the amount of total labeled DNA in the reaction.

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