

The Mutagenesis Proteins UmuD' and UmuC Prevent Lethal Frameshifts While Increasing Base Substitution Mutations

Nina Bacher Reuven,[†] Guy Tomer,[†] and Zvi Livneh*

Department of Biological Chemistry
Faculty of Biochemistry
The Weizmann Institute of Science
Rehovot 76100
Israel

Summary

Error-prone DNA repair consists of replicative filling-in of DNA gaps carrying lesions. We have reconstituted *E. coli* SOS error-prone repair using purified DNA polymerase III holoenzyme, SSB, RecA, UmuD', a UmuC fusion protein, and a gap lesion plasmid. In the absence of UmuDC, or without SOS induction, replication skips over the lesion, forming mostly one-nucleotide deletions. These cause translational frameshifts that usually inactivate genes. UmuD' and UmuC, in the presence of RecA and SSB, stimulate translesion replication and change its mutagenic specificity such that deletions are prevented and base substitutions are increased. This results in mutagenic but nondetrimental gap repair and provides an effective mechanism for generating genetic variation in bacteria adapting to environmental stress.

Introduction

Genomic DNA is continuously subjected to damage by internal and external agents, such as reactive oxygen species or sunlight, and by spontaneous decay (e.g., depurination). The DNA lesions produced interfere with replication and with gene expression, and they must be removed by DNA repair enzymes in order to enable proper function of DNA. When unrepaired lesions are replicated, they give rise to mutations due to their mis-coding potential (Friedberg et al., 1995). This is of major interest from the human disease standpoint, since the formation of mutations in critical target genes (oncogenes and tumor suppressor genes) leads to cancer. It has been estimated that most human cancers are caused by unrepaired DNA lesions (Sancar, 1994). A broad class of DNA lesions, including UV light-induced pyrimidine cyclobutyl dimers or 6-4 adducts, abasic sites, or DNA adducts produced by certain drugs, such as cisplatin, interrupt DNA replication, leading to the formation of single-stranded regions. Such structures, ssDNA regions carrying damaged bases (gap lesion structures), cannot be repaired by the regular excision repair pathways, because that would lead to a double-strand break, which is highly lethal. The emergency tolerance strategy adopted for such cases is to repair (fill in) the gap without removing the damaged base. This converts the single-stranded region back into a duplex

structure, thus restoring DNA continuity and reducing the risk of chromosome breakage. Excision repair mechanisms might then have, at a later stage, a second chance to remove the lesion (Livneh et al., 1993; Friedberg et al., 1995).

Two general mechanisms are known for filling-in of gap lesion structures. Recombinational repair relies on the homologous fully replicated sister chromatid to provide a DNA segment that is patched across the lesion. This process is fundamentally error-free and is a major repair function in *E. coli* (Kowalczykowski et al., 1994; Eggleston and West, 1996). The second strategy consists of filling-in of the gap lesion by a DNA polymerase. This mechanism is mutagenic because polymerases tend to incorporate incorrect nucleotides opposite DNA lesions. In *E. coli*, this process, which is the paradigm for genetically regulated mutagenesis, is under tight regulation by the SOS stress response and requires specific inducible proteins. Its major outcome is a dramatic increase in mutations associated with DNA damage. It was termed error-prone DNA repair, SOS repair, or SOS mutagenesis (referring to its outcome), or translesion replication (referring to its mechanism; reviewed in Livneh et al., 1993; Walker, 1995). Two suggestions were offered to explain the function of such a system in *E. coli*. First is the repair of DNA gaps (opposite lesions) on which recombination cannot act (e.g., overlapping daughter strand gaps). The price of this repair is an increase in mutation frequency. The second is the facilitated adaptation of cell populations to environmental stress conditions, via an inducible mutagenesis mechanism (Radman, 1975; Witkin, 1976; Bridges, 1978; Echols, 1981). The latter function is particularly intriguing because it implies an active mode of evolution (Echols, 1981).

In *E. coli*, the major tolerance mechanism toward unrepaired lesions is recombinational repair. In contrast, recombinational repair in mammals is less active (Friedberg et al., 1995), perhaps because of the large proportion of repetitive sequences in the mammalian genome, which increases the danger of undesired gross rearrangements. This leaves translesion replication as the major candidate for tolerance of unrepaired lesions in mammals. The scarcity of knowledge on mammalian tolerance of DNA damage underscores the importance of elucidating similar mechanisms in model organisms such as bacteria and yeast.

Based on genetic analysis, SOS mutagenesis in *E. coli* requires DNA polymerase III (Bridges et al., 1976; Brotcorne-Lannoye et al., 1985), which is the replicative DNA polymerase, as well as three SOS-inducible proteins: RecA, UmuD', and UmuC. RecA is a multifunctional protein, known to be the major recombinase in *E. coli* (Roca and Cox, 1990), but its function in SOS mutagenesis is not directly related to recombination. RecA fulfills three roles in SOS mutagenesis, of which two are regulatory (Witkin, 1991): First, it activates the SOS stress response by promoting the cleavage of the LexA repressor. This induces the expression of the mutagenesis-specific proteins UmuD and UmuC. Second,

* To whom correspondence should be addressed.

[†] These authors contributed equally to this work.

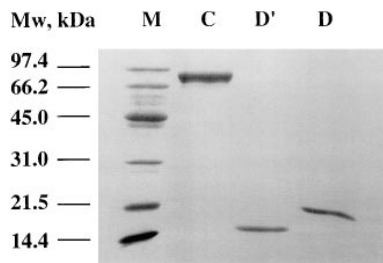


Figure 1. Gel Analysis of the Purified Umu Proteins
The purified UmuD (marked D), UmuD' (D'), and MBP-UmuC (C) proteins were analyzed by 12% PAGE, followed by visualization by Coomassie-blue staining. M, molecular weight markers.

it promotes the posttranslational cleavage of UmuD to UmuD', the active form in mutagenesis (Burckhardt et al., 1988; Nohmi et al., 1988; Shinagawa et al., 1988). In addition, RecA has been suggested to have a third, presumably direct, role in the mutagenic process (Dutreix et al., 1989; Sweasy et al., 1990). UmuD' and UmuC are specifically required for SOS mutagenesis (Kato and Shinoura, 1977). A pioneering study by Rajagopalan et al. (1992) indicated that UmuD' and UmuC act as bypass factors and increase translesion replication by pol III holoenzyme. However, the further utilization of that experimental system was hampered by the difficulty in obtaining purified active UmuC (Woodgate et al., 1989). Here we report the *in vitro* reconstitution of a robust SOS translesion replication reaction, using DNA polymerase III holoenzyme, SSB, RecA, UmuD', and a UmuC fusion protein. Moreover, we show that UmuD' and UmuC prevent lethal deletion mutations while increasing nonlethal base substitutions.

Results

Based on *in vivo* studies, and on the pioneering study of Rajagopalan et al. (1992), SOS mutagenesis results from a translesion replication reaction that requires DNA polymerase III, RecA, UmuD', and UmuC (Livneh et al., 1993; Walker, 1995; Woodgate and Levine, 1996). The major obstacles in the attempts to study the mechanism of SOS mutagenesis using an *in vitro* reconstituted system were the inability to obtain reproducibly purified and active UmuC (Woodgate et al., 1989) and the difficulty in constructing an appropriate DNA substrate. We have overproduced and purified UmuD, UmuD', and UmuC as soluble proteins fused to a portion of the maltose-binding protein (MBP). Once purified, the MBP tag was removed from the UmuD and UmuD' fusion proteins using factor Xa protease (Figure 1). The MBP-UmuC protein was found to be resistant to cleavage and was used in subsequent experiments as the fusion protein (Figure 1). It was found to bind ssDNA (data not shown), consistent with previous reports that UmuC binds ssDNA (Petit et al., 1994; Bruck et al., 1996).

A critical component in reconstitution of SOS translesion replication was the DNA substrate used, which consisted of a gapped plasmid carrying a site-specific lesion

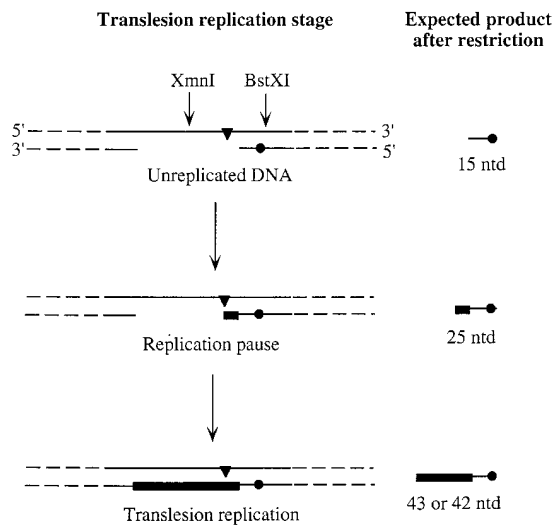


Figure 2. Outline of the Translesion Replication Assay Using a Gap Lesion Plasmid

The replication products are shown before (left side) and after (right side) cleavage with restriction nucleases XmnI and BstXI. Only a portion of the plasmid is shown. The ssDNA gap was 350 nucleotides long, and the primer terminus was located 11 nucleotides upstream of the lesion. Closed triangle, synthetic abasic site; closed circle, radiolabeled phosphate; see text for details.

located within a single-stranded region of 350 nucleotides (Figure 2). Such a construct serves as a good substrate for replication by the multisubunit pol III holoenzyme, and for binding by RecA and SSB. A method for the preparation of these gap lesion plasmids was recently developed in our laboratory (Tomer and Livneh, submitted). When a DNA polymerase was added to the substrate, the 3' terminus was extended, and this was monitored by a gel assay, after cleaving the DNA with two restriction nucleases: BstXI, which cleaves just upstream to the internal ³²P radiolabel, and XmnI, which cleaves downstream to the lesion (Figure 2). This cleavage was introduced in the assay in order to reduce the sizes of radiolabeled replication products, and thus to increase resolution. A synthetic abasic site was used as a model lesion. Abasic sites, which are very common lesions in DNA, are known to inhibit DNA replication and give rise to mutations via the UmuD' and UmuC pathway (Loeb and Preston, 1986).

Incubation of the gap lesion plasmid with DNA polymerase III holoenzyme resulted in inhibition of replication at the nucleotide preceding the lesion (25-nucleotide-long product), but translesion replication was also observed (Figure 3, lane 2). This is consistent with previous results on the ability of pol III holoenzyme to bypass a native or synthetic abasic site, unassisted by other proteins (Hevroni and Livneh, 1988; Tomer and Livneh, submitted). Addition of RecA led to a 2- to 3-fold increase in translesion replication (Figure 3, lane 3). This demonstrates that RecA directly stimulates translesion replication. SSB, which melts out secondary structures and was recently found to interact with a UmuC homolog (MucB; Sarov-Blat and Livneh, 1998), also increased translesion replication 2- to 3-fold (Figure 3, lane 4),

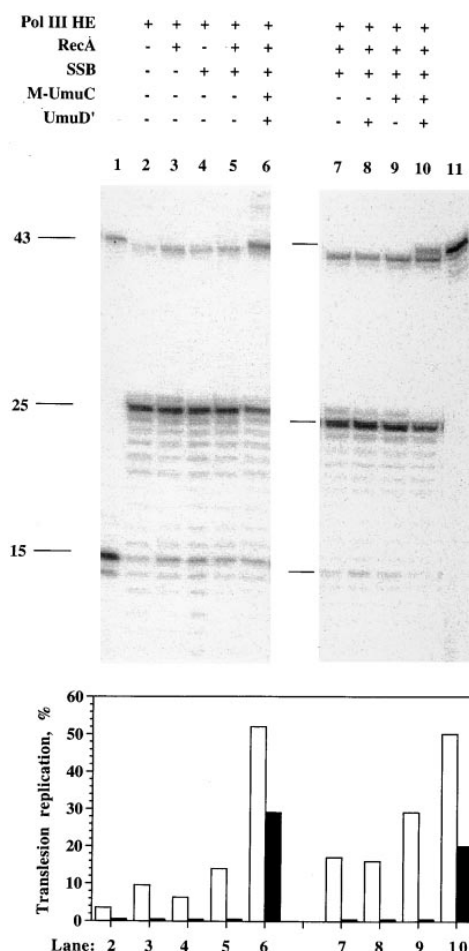


Figure 3. Effects of RecA, SSB, UmuD', and the Fusion UmuC Protein on Translesion Replication by DNA Polymerase III Holoenzyme. Translesion replication was carried out with pol III holoenzyme and the gap lesion plasmid GP21, in the presence of RecA, SSB, UmuD', and the fusion UmuC protein (M-UmuC) as indicated, at 37°C for 8 min. The samples were deproteinized, fractionated by urea-PAGE, and visualized and quantified by phosphorimaging. The details are presented under Experimental Procedures. (Top) Phosphorimager of the gel. Lane 1 contains markers for unreplicated DNA (15-mer) and fully replicated DNA (43-mer). They were obtained by restriction of nonreplicated plasmid or by ³²P-end labeling of a synthetic 43-mer with the corresponding DNA sequence, respectively. Lane 11 contains the marker for fully replicated products (43-mer). (Bottom) quantitation of the results shown in the phosphorimager. Open bars, total translesion replication; closed bars, full translesion replication, represented by the 43-mer only.

consistent with previous results (Livneh, 1986a; Tomer, Reuven, and Livneh, submitted). When both RecA and SSB were added, stimulation of translesion replication was only marginally higher than with either of the proteins alone, reaching an effect of 3- to 4-fold over pol III holoenzyme alone (Figure 3, lanes 5 and 7). When UmuD' and the UmuC fusion protein were added as well, forming a five-protein reaction, there was an additional 3-fold increase in translesion replication, reaching 50%. Most importantly, when all five proteins were present, an additional bypass product, one nucleotide

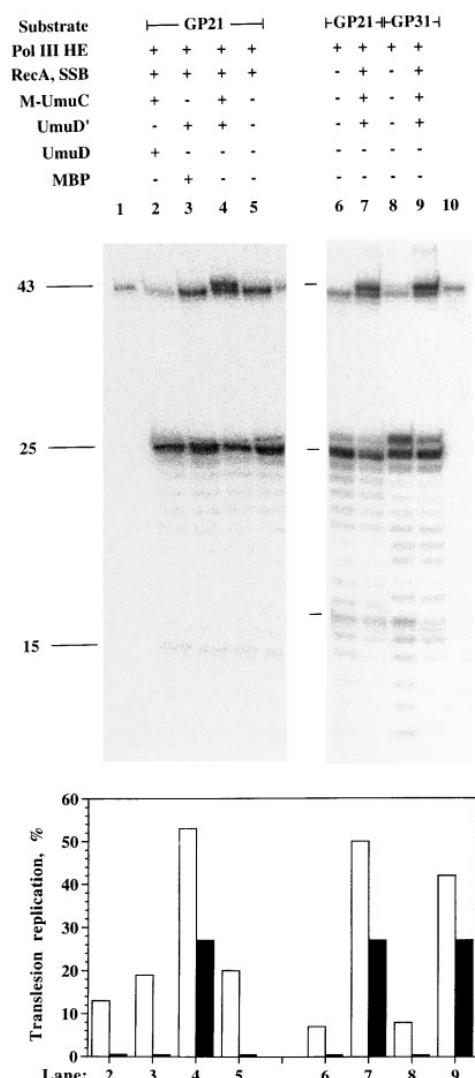


Figure 4. Characteristics of SOS Translesion Replication. Reactions were carried out as described in the legend to Figure 3, with the proteins and DNA substrates as indicated. (Top) Phosphorimager of the gel. Lane 2 contains UmuD instead of UmuD'; 3, the MBP tag was added instead of the fusion MBP-UmuC protein. Lanes 6 and 7, and 8 and 9 contain side-by-side reactions carried out with substrates GP21 and GP31, respectively. (Bottom) Quantification of the results shown in the phosphorimager. Lanes 1 and 10 contain markers for the fully replicated DNA (a synthetic 43-mer). Open bars, total translesion replication; closed bars, full translesion replication, represented by the 43-mer only.

longer, was formed (Figure 3, lanes 6 and 10). This product was not formed in the absence of either the UmuC fusion protein, or UmuD' (Figure 3, lanes 8 and 9, respectively), nor was it formed when UmuD was used instead of UmuD' (Figure 4, lane 2), nor when MBP was used instead of the MBP-UmuC fusion protein (Figure 4, lane 3). By comparison to the migration of a DNA size marker, the longer product was found to be 43 nucleotides long, representing the full-length translesion replication product (Figures 3 and 4). As we demonstrate below, the shorter product (the 42-nucleotide-long band) was formed when the polymerase skipped over the lesion, that is, it

Table 1. Requirements for Reconstituted SOS Translesion Replication

Component Omitted	Overall Bypass (%)	Bypass without Skipping (%)
None	50	35
Pol III HE	<0.5	<0.5
SSB	28	<0.5
RecA	22	<0.5
SSB, RecA	17	<0.5
UmuD'	30	<0.5
M-UmuC	13	<0.5
UmuD', M-UmuC	14	<0.5

Translesion replication of substrate GP21 was carried out for 8 min with pol III holoenzyme, SSB, RecA, UmuD', and M-UmuC (the UmuC fusion protein), or in the absence of the indicated components. Reaction conditions and analysis were as described under Experimental Procedures. The table shows the quantification of phosphorimages similar to that presented in Figure 3.

did not incorporate a nucleotide opposite the synthetic abasic site during the gap-filling replication (see also Figure 7).

To determine whether the reconstituted translesion replication reaction occurs in another DNA sequence context, we used the gap lesion plasmid GP31, in which the sequence surrounding the lesion was changed. As can be seen in Figure 4 (lanes 6–10), similar results were obtained with the two substrates. Addition of SSB, RecA, UmuD', and the UmuC fusion protein stimulated replication by pol III holoenzyme through the lesion on both substrates, and a 43-nucleotide band, not observed with pol III holoenzyme alone, was formed (Figure 4, lanes 6–10). Table 1 shows the requirements of the reaction. It is clear that omission of any of the proteins from the reaction mixture results in loss of the ability to obtain the full-length product. Thus, all four proteins, RecA, UmuD', the UmuC fusion protein, and SSB, are each required in the reconstituted reaction. A time course of translesion replication with pol III alone or with the addition of SSB, RecA, UmuD', and the UmuC fusion protein is shown in Figure 5. As can be seen, the reconstituted system is robust and performs highly effective translesion replication leading to 70% bypass in 12 min. The major bypass product is 43 nucleotides long, representing full replication, without skipping over the lesion.

In order to establish the replication specificity across the synthetic abasic site, we have amplified the replication products using PCR and determined the DNA sequence at the site of the lesion. The results of these experiments are shown in Table 2. All of the 26 isolates obtained during replication by pol III holoenzyme alone contained small deletions at the site corresponding to the lesion in the original substrate. Of the 26 mutants, 22 were one-nucleotide deletions (85%). No base substitutions were observed. Thus, although pol III holoenzyme can replicate through the synthetic abasic site, it does so by skipping the lesion. DNA sequence analysis of bypass products synthesized in the fully reconstituted system revealed that in this case, too, all mutations were targeted to the lesion. However, a dramatic change was observed in mutation type. The majority of mutations were now base substitutions (63%), implying insertion

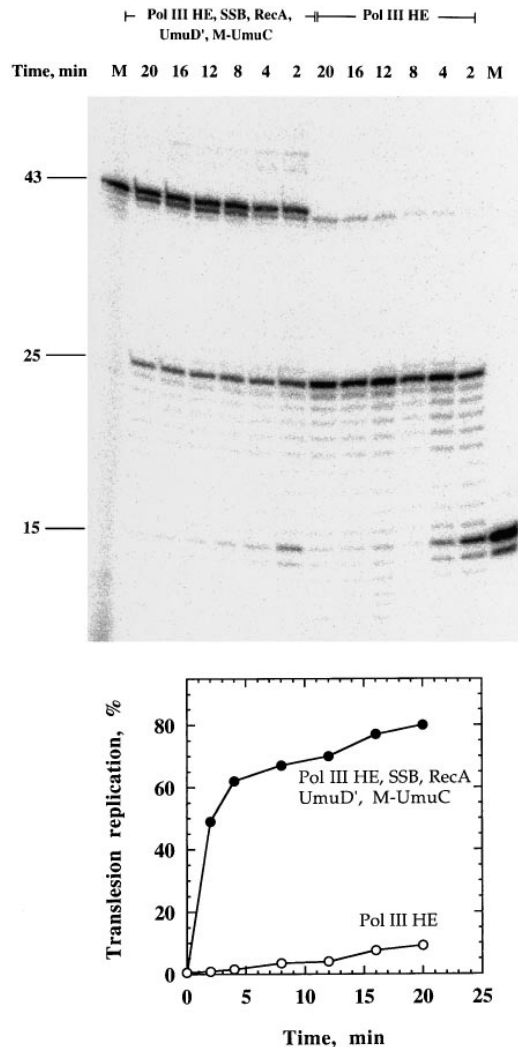


Figure 5. Time Course of In Vitro Reconstituted SOS Translesion Replication

Translesion replication of substrate GP21 was carried out with pol III holoenzyme alone, or in the presence of SSB, RecA, UmuD', and M-UmuC for the indicated time periods. The details are presented under Experimental Procedures.

(Top) Phosphorimage of the gel. The M lanes show size markers for unreplicated DNA (15-mer; right M lane) and for completely replicated DNA (left M lane).

(Bottom) Quantification of the results shown in the phosphorimage. Open circles, translesion replication with pol III holoenzyme alone; closed circles, translesion replication with pol III holoenzyme, SSB, RecA, UmuD', and the fusion UmuC protein (M-UmuC).

of a nucleotide opposite the lesion during the translesion replication reaction. Most base substitutions had an A inserted opposite the abasic site, in agreement with in vivo results on the mutagenic specificity of abasic sites (Kunkel, 1984; Lawrence et al., 1990). These results confirm the identity of the 42- and 43-nucleotide-long DNA products seen in the gel assay as representing a one-nucleotide deletion and full-length bypass product, respectively. In addition, they reveal a function of the SOS-induced proteins, as antideletion agents.

Previous in vivo studies have established that under

Table 2. Analysis of Mutations Formed during *In Vitro* Translesion Replication

Mutation Type	Translesion Replication Reaction Components	
	Pol III HE	Pol III HE, SSB, RecA UmuD', M-UmuC
Base substitution		
A	—	11
G	—	5
T	—	1
C	—	—
Total base substitutions	— (0%)	17 (<u>63%</u>)
Deletions		
-1	22 (85%)	10
-2	3	—
-3	1	—
Total deletions	26 (<u>100%</u>)	10 (37%)
Total mutants analyzed	26	27

Translesion replication of substrate GP21 was carried out in the presence of the indicated proteins, after which the newly synthesized strand was amplified, cloned into plasmid pUC18, and introduced into *E. coli* cells. The table shows the DNA sequence opposite the lesion obtained for individual clones. The details are presented under Experimental Procedures. The values in parentheses represent the percentage of the particular mutation type. The most abundant type of mutation is underlined.

SOS conditions abasic sites cause primarily base substitutions, with insertion of a dAMP residue being the major event (Kunkel, 1984; Lawrence et al., 1990). However, in these studies mutational specificity was not examined in noninduced cells or in the absence of the Umu proteins. We utilized our gap lesion plasmids used in the *in vitro* experiments in order to examine the *in vivo* mutagenicity of the synthetic abasic site. The experimental protocol involved transformation of uninduced or SOS-induced cells with gap lesion plasmids that were not subjected to any treatment. The plasmids can be maintained in the host cells only after the gap is repaired *in vivo*. In the absence of a homologous double-stranded DNA, the only known mechanism to repair the gap is translesion replication. Thus, the number of transformants obtained is a reflection of the efficiency of *in vivo* translesion replication operating on the plasmid. The survival of a gapped plasmid carrying a lesion is defined by the efficiency in which it transforms a particular *E. coli* strain, compared to a gapped plasmid without a lesion. Plasmids were isolated from cultures of transformed cells, and the DNA sequence in the region that originally carried the lesion was determined.

The results of these experiments are presented in Table 3 and in Figure 6. The survival of the gap lesion plasmid GP21 in the noninduced "wild-type" cells was 0.9% compared to a gapped plasmid without a lesion, and it increased to 5.0% in SOS-induced cells (Table 3, bottom). The survival of another gapped plasmid, GP31, was increased by SOS induction from 0.6% to 2.1%. Each plasmid recovered contained a mutation, and all mutations were targeted to the lesion. In the absence of SOS induction, 68% of the mutations in plasmid GP21 were small deletions. Interestingly, when a nucleotide was inserted opposite the lesion in the absence of SOS

induction, it was a C, not an A as in SOS-induced cells. The picture changed dramatically when SOS-induced cells were examined: 76% of the mutations were base substitutions, consisting mostly of insertion of A opposite the lesion. The difference in mutational specificity was even more pronounced with plasmid GP31: 100% of the mutations in noninduced cells were small deletions, whereas in SOS-induced cells 71% of the mutations were base substitutions, mostly A (Table 3). These results are consistent with the *in vitro* results presented above and indicate that SOS induction leads to a suppression of small deletions, and to the promotion of base substitutions (Figure 6).

In order to examine whether the UmuD and UmuC proteins were involved in this process *in vivo*, we repeated the transformation experiments with an uninduced and SOS-induced Δ umuDC strain. Plasmid survival was 0.7% in the noninduced Δ umuDC cells, but unlike in UmuDC⁺ cells it was unchanged after SOS induction (Table 3). Thus, increased survival of the gap lesion plasmid in SOS-induced cells was totally dependent on the Umu proteins. The DNA sequence analysis revealed that in the noninduced Δ umuDC cells 86% of the mutations were small deletions, similar to the situation in noninduced UmuDC⁺ cells. The predominance of small deletions occurred also when the Δ umuDC cells were SOS-induced (Table 3). These results strongly suggest that the UmuD' and UmuC proteins function to suppress small deletions, a lethal type of mutation, and promote base substitutions, a mild type of mutation.

Discussion

The *in vitro* reconstitution of SOS translesion replication establishes that SOS mutagenesis, at least in the case of abasic sites, proceeds via UmuD', UmuC, RecA, and SSB-stimulated translesion replication, confirming the basic results of Rajagopalan et al. (1992). We have previously shown that DNA polymerase III holoenzyme can replicate "blocking" lesions at high efficiency, unassisted by any other protein (Livneh, 1986b; Hevroni and Livneh, 1988; Tomer and Livneh, submitted). These results presented an apparent paradox: *In vitro* bypass did not require UmuD', UmuC, and RecA, whereas *in vivo* bypass did require these proteins. This paradox seems now to be resolved: Pol III holoenzyme can indeed bypass a synthetic abasic site unassisted, but it does so in a "sloppy" way, by skipping over the lesion, and producing mostly -1 deletions (Figure 7). Such deletions cause translational frameshifts, which usually render genes nonfunctional. The UmuD' and UmuC proteins stimulate translesion replication and at the same time cause a dramatic change in its mutagenic specificity: They prevent the lethal frameshift mutations while increasing base substitution, a mild type of mutation.

The reason that the polymerase skips over the lesion in the absence of the SOS proteins might be the stabilization of a misaligned abasic site (Figure 7). Support for such a model comes from the *in vivo* experiments that show the identity of nucleotides inserted opposite the lesion in the absence of SOS induction. In contrast to SOS conditions, under which an A is inserted opposite

Table 3. In Vivo Mutational Specificity of Gapped Plasmids Containing Site-Specific Synthetic Abasic Sites

Mutation Type or Plasmid Survival	Substrate GP21				Substrate GP31	
	AB1157wt		WBY100 Δ umuDC		AB1157wt	
	-SOS	+SOS	-SOS	+SOS	-SOS	+SOS
Base Substitution						
A	—	14	—	—	—	15
G	—	1	—	—	—	1
T	—	1	—	—	—	—
C	8	3	3	8	—	—
Total base substitutions	8 (32%)	19 (<u>76%</u>)	3 (14%)	8 (36%)	— (0%)	16 (<u>80%</u>)
Deletion						
-1	7	2	11	8	18	4
-2	4	3	5	2	5	—
-3	4	1	3	—	2	—
Others	2	—	—	4	—	—
Total deletions	17 (<u>68%</u>)	6 (24%)	19 (<u>86%</u>)	14 (64%)	25 (100%)	4 (20%)
Total mutants analyzed	25	25	22	22	25	20
Plasmid survival	0.9%	5.0%	0.7%	0.7%	0.6%	2.1%

The gap lesion plasmids GP21 and GP31 were introduced into SOS-induced or noninduced *E. coli* cells, as indicated. Plasmid survival is calculated by dividing the number of transformants by that obtained with the corresponding control gapped plasmid without the lesion. The table shows the DNA sequence opposite the lesion obtained for individual clones. The details are presented under Experimental Procedures. The values in parentheses represent the percentage of the particular mutation type. The most abundant type of mutation is underlined.

the lesion, in the absence of SOS, when a nucleotide was inserted opposite the lesion, it was a C (Table 3). This nucleotide is complementary to the nucleotide next and downstream to the lesion in substrate GP21 (Figure 7). Such a phenomenon can be explained as follows: The polymerase skips the lesion and copies the next nucleotide, but in some cases, before proceeding to the next polymerization step, the abasic site flips back in, and the polymerase again copies the nucleotide next to the abasic site and proceeds in replication (Figure 7). SOS proteins might prevent misalignment of the lesion, possibly through the formation of a stable multiprotein-

DNA complex. The generation of -1 frameshifts during translesion replication by pol III holoenzyme may be related to the propensity of its catalytic α subunit to produce -1 frameshift mutations during a gap-filling replication on undamaged templates (Mo and Schaaper, 1996) and to a Umu-independent branch of mutagenesis associated with 2-acetylaminofluorene adducts (Napolitano et al., 1997). A similar behavior was observed for mammalian DNA polymerase β (Kunkel, 1990; Efrati et al., 1997).

A striking feature of the translesion replication system is its very high effectiveness: 70% of the gap lesions were filled in within 12 min (Figure 5). This suggests that translesion replication is potentially an effective way to repair gap lesion structures. Its efficiency in acting on chromosomal gap lesion structures in vivo may be limited by at least two factors: First, inhibition of translesion replication by DNA damage-binding proteins through direct binding to the gap lesion (Paz-Elizur et al., 1997). Second, competition from recombinational repair, which provides an error-free alternative for filling in gap lesion structures. Indeed, it was reported that overproduction of UmuD'C inhibited recombinational repair (Sommer et al., 1993; Boudsocq et al., 1997).

SOS translesion replication was suggested to act both as a cellular DNA repair mechanism, as well as an inducible mutator (Radman, 1975; Witkin, 1976; Bridges, 1978; Echols, 1981). The suppression of deletion mutations is important for both purposes: there is no point in repairing a gap if the result will be a lethal deletion, and it is inefficient to generate genetic variation by promoting harmful deletion mutations. In both cases, base substitution is a better mutation, because it is usually milder in its biological consequences.

The yeast *S. cerevisiae* is similar to *E. coli* with regard to dealing with gap lesion structures by both an error-free recombinational repair mechanism and translesion

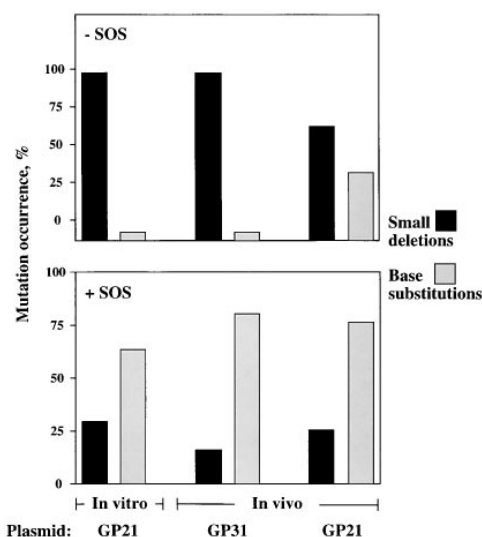


Figure 6. A Comparison of Mutations Produced with and without SOS Induction

The data was taken from Tables 2 and 3.

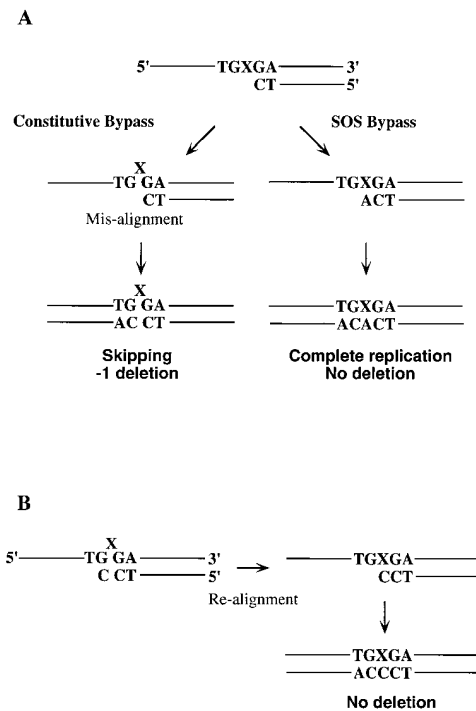


Figure 7. A Model Describing SOS and Constitutive Modes of Translesion Replication

(A) Constitutive bypass involves misalignment of the lesion, resulting in skipping by the polymerase across the lesion, and the formation of a -1 deletion. This is a drastic mutation, which usually leads to inactivation of genes. Under SOS stress conditions, translesion replication occurs without skipping, resulting in insertion of a nucleotide opposite the lesion. This base substitution is usually a tolerable type of mutation. The DNA sequence is from the gap lesion plasmid GP21.

(B) A minor bypass pathway without deletion in the absence of SOS induction. After copying the nucleotide past the lesion in the misaligned state, the lesion realigns, and replication proceeds. This leads to complete translesion replication, with the nucleotide present opposite the lesion being complementary to the nucleotide past the lesion.

replication. Unlike *E. coli*, it has a specialized DNA polymerase for the translesion replication reaction (DNA polymerase ζ , product of the *REV3* and *REV7* genes; Nelson et al., 1996b), and a dCMP nucleotidyl transferase, product of the *REV1* gene (Nelson et al., 1996a). Mammalian cells do not seem to use homologous recombination extensively (Friedberg et al., 1995), probably due to the high content of repetitive sequences in the mammalian genome. Nevertheless, the DNA is fully replicated, and cells divide even when DNA repair is not complete, and damage persists in the genome (Friedberg et al., 1995). This suggests that translesion replication may be an important recovery mechanism in the response of mammalian cells to DNA damage and underscores the importance of elucidating the molecular mechanisms of translesion replication in model organisms such as *E. coli* and yeast.

Experimental Procedures

Overexpression and purification of UmuD', UmuD, and UmuC fused to MBP. The fragments used for cloning *umuD'*, *umuD* and *umuC*

into the expression vector pMAL-c2 (New England Biolabs) were prepared by PCR, using plasmid pSE117 as a template (Marsh and Walker, 1985). The 5' primers used for PCR corresponded to the 5' end of the respective genes as follows: *umuC* primer, 5'-ATGTTTGC CCTCTGTGATGTAACGCG-3'; *umuD* primer, 5'-ATGTTGTTTATCA AGCTGCGGATC-3'; and *umuD'* primer, 5'-GGCTTTCCTTACC GCAGCAG-3'. The 3' primers contained an EcoRI site at their 5' end to facilitate cloning and continued with sequences complementary to the 3' end of the genes, including the stop codons as follows: *umuC* primer, 5'-CCGGAATTCCTTTATTTGACCCTCAGTAAATC-3'; and *umuD/D'* primer, 5'-CGGAATTCATCAGCGCATCGCCTTA ACG-3'. The PCR products were cut with EcoRI and cloned into pMAL-c2 at the XmnI and EcoRI sites. The expression products from these vectors are fusion proteins with a 42.7 kDa maltose-binding protein (MBP) portion at the N terminus. A cleavage site for Factor Xa protease is situated such that cleavage with this protease liberates the native Umu proteins without adding additional amino acids.

Vectors expressing UmuD or UmuD' were transformed into WBY11, a $\Delta recA \Delta umuDC$ strain. Cells were grown to OD_{595} 0.4–0.6 at 30°C in LB supplemented with 0.2% glucose. IPTG was added to 0.3 mM, and cells were grown for an additional 3 hr. The cells were harvested, resuspended in buffer A (20 mM Tris-HCl [pH 7.5], 10% glycerol, 200 mM NaCl, 1 mM EDTA, 1 mM DTT), and quick-frozen. The cells were then thawed, sonicated, and centrifuged at 180,000 g for 1 hr at 4°C. The supernatant was loaded on an amylose column, and fusion proteins eluted with buffer A containing 10 mM maltose. The fusion proteins were cleaved with 0.3% (w/w) Factor Xa at 4°C for 20 hr. For UmuD' purification, the mixture was loaded onto a Sephacel G-75 gel filtration column (to separate residual uncleaved fusion protein), and fractions containing UmuD' were pooled. This step was unnecessary for UmuD purification because cleavage of its fusion protein was complete. The fractions were dialyzed to bring the salt concentration to 80 mM NaCl. The proteins were then loaded on a DEAE Sephacel column, and proteins eluted using a gradient of 80–500 mM NaCl. UmuD eluted at 195 mM NaCl, and UmuD' at 150 mM NaCl. The purity of UmuD and UmuD' was >90%, as judged by Coomassie blue-stained gels (Figure 1).

Extracts were prepared from cells overexpressing MBP-UmuC as for UmuD, except that a cocktail of protease inhibitors (Sigma) was added to the cells before sonication. Cell extract was loaded on a phosphocellulose P-11 column using buffer A. The protein was eluted using a gradient of 0.2–1.0 M NaCl. The fusion protein eluted at 500 mM NaCl. Fractions containing the fusion protein were collected and loaded onto an amylose column. The fusion protein was eluted from the amylose column with buffer A containing 10 mM maltose. The purity of MBP-UmuC was 90%, as judged by Coomassie blue-stained gels (Figure 1).

Other Proteins

Pol III holoenzyme (Cull and McHenry, 1995), SSB (Lohman and Overman, 1985), and RecA (Cox et al., 1981) were purified as described, except that a phosphocellulose purification step was added for RecA. Restriction nucleases, T4 DNA ligase, and T4 polynucleotide kinase were from New England Biolabs. T7 gp6 exonuclease was from Amersham.

DNA Substrates

The preparation of the gapped plasmid carrying a site-specific lesion was recently described (Tomer and Livneh, submitted). In brief, plasmid pSKSL (3361 bp), a derivative of plasmid pBluescript II SK(+), was cleaved with restriction nucleases BstXI and BsaI and ligated to a synthetic gapped duplex oligonucleotide whose termini were complementary to those of the cleaved plasmid. The DNA sequences of the gapped duplexes, which carried a site-specific synthetic abasic site (X), were prepared by annealing the following sequences: gapped duplex GD21, 5'-ACGCAACGAAGTGATCCCGTCTGACTGXGAAAACCTGGGCTACTTGAACCAGACCG-3', 3'-GTTGCTTCACTAAGG-5', and 3'-CCGATGAACCTGGTC*-5'; and gapped duplex GD31, 5'-ACGCAACGAAGTGATTCCTGGCGTTACCCXACTTAATCGCGCTACTTGAACCAGACCG-3', 3'-GTTGCTTCACTAAGG-5', and 3'-CCGATGAACCTGGTC*-5'. (The asterisks mark a 5'-³²P radiolabeled phosphate group, and the XmnI cleavage

site is in bold). The desired gapped plasmids, termed GP21 and GP31, respectively, were gel-purified, and the gaps were extended to a size of 350 nucleotides using the T7 gp6 5'→3' exonuclease, as described before (Tomer, Reuven, and Livneh, submitted). Undamaged control plasmids were prepared similarly, except that the oligonucleotides had a G instead of the abasic site. All oligonucleotides were synthesized and purified by the Synthesis Unit of the Biological Services Department at the Weizmann Institute of Science. Oligonucleotides containing the synthetic abasic site analog were synthesized similarly using dSpacer CE phosphoramidite (Glen Research) as a building block. The abasic site analog is a modified tetrahydrofuran moiety, which is a stable analog of 2'-deoxyribose in the abasic site. It has a hydrogen instead of a hydroxyl residue on 1' carbon of the deoxyribose ring (Takeshita et al., 1987).

Translesion Replication Assay

The translesion replication reaction mixture (25 μ l) contained 20 mM Tris-HCl (pH 7.5), 8 μ g/ml bovine serum albumin, 5 mM DTT, 0.1 mM EDTA, 4% glycerol, 1 mM ATP, 10 mM MgCl₂, 0.5 mM each of dATP, dGTP, dTTP, and dCTP, 0.1 μ g (2 nM) gapped plasmid and 1 nM pol III holoenzyme, 0.6 μ M SSB, 4 μ M RecA, 2.5 μ M UmuD' or UmuD, and 0.23 μ M M-UmuC or MBP. Reactions were carried out at 37°C for 2–20 min, after which they were terminated by adding SDS to 0.2%, EDTA to 20 mM, and NaCl to 200 mM and heat-inactivated at 65°C for 10 min. The proteins were digested with 0.4 mg/ml proteinase K at 37°C for 1 hr, after which the DNA was extracted with phenol-chloroform and ethanol-precipitated. The DNA was digested with XmnI (3 U/tube) at 37°C for 2 hr. Then, 5 U of BstXI was added, and incubation continued at 55°C for another 2 hr. The DNA was fractionated by electrophoresis on 15% polyacrylamide gels containing 8 M urea. Gels were run at 1500–2000 V for 2–3 hr, after which they were dried, and visualized and quantified using a Fuji BAS 2000 phosphorimager. The extent of translesion replication was calculated by dividing the amount of bypass product by the amount of extended primer.

DNA Sequence Analysis of Translesion

Replication Products

DNA (100 ng) from the translesion replication assay was processed as indicated above, until the XmnI cleavage step. Instead of cleaving with XmnI, the DNA was cut with HindIII (20 U/tube), which cuts the plasmid at a single site 867 bp from the abasic site. DNA was then digested with 2 U of S1 nuclease in a final volume of 32 μ l for 30 min at 30°C to degrade unextended ssDNA template regions. The reaction was stopped by adding 128 μ l of 25 mM EDTA and heating at 80°C for 10 min. The mixture was extracted with phenol:chloroform (1:1), then chloroform. Twenty microliters of this DNA sample was linearly amplified in a 50 μ l PCR reaction using 1 U Taq DNA polymerase (Appligene), 200 μ M dNTPs, and 50 pmol of primer #1, containing an EcoRI site at its 5' end, and complementary to the newly synthesized strand. The sequence of primer #1 was 5'-GAGAA TTCGCAATGATACCGCCGCAACGAAGTG-3', and its 3' end was 16 nucleotides upstream from the abasic site. The PCR mixture was heated to 95°C for 1 min, 56°C for 5 min, and 72°C for 3 min. This was followed by 39 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 2 min, and extension at 72°C for 3 min. The PCR products were fractionated on a 0.8% low-melting agarose gel, and the 913-nucleotide-long PCR products in gel slices were subjected to a second round of regular PCR. Twenty microliters of melted gel samples containing the DNA was amplified with primer #1 and primer #2, which contains a BamHI site at its 5'. The sequence of primer #2, complementary to nucleotides 466–449 of plasmid pSKSL, was 5'-CGGGATCCGAAGGTGGAGGAAGGTG-3'. The PCR program used was the same as above, except that only 35 cycles and 2 min extension times were used. This procedure yielded 273 bp PCR products. A control amplification protocol performed with the gapped plasmid that had not undergone a translesion replication reaction gave no PCR products, indicating that the S1 nuclease treatment was effective in eliminating nonreplicated DNA molecules. The 273 bp PCR products were gel-purified, digested with BamHI and EcoRI, and cloned into pUC18 at those sites. Individual transformants were picked, and their plasmid contents extracted and subjected to automated DNA sequence analysis, performed by the

Biological Services Department in our institute. In this procedure, each translesion replication event was scored, and there was no selection for specific mutagenic events.

In Vivo Mutagenesis of Gap Lesion Plasmids

The gap lesion plasmids GP21 or GP31, each carrying a site-specific synthetic abasic site, and the respective control plasmids GP20 or GP30 without a lesion were used to transform competent *E. coli* AB1157 *argE3 hisG4 leuB6 Δ (gpt-proA)62 thr-1 ara-14 galK2 lacY1 mtl-1 xyl-5 thi1 tsx-33 rpsL31 supE44* or its isogenic Δ umuDC derivative, WBY100. For experiments under SOS-induction, the cells were UV-irradiated at 30 Jm⁻², followed by a 30 min incubation period at 37°C, to allow expression of SOS functions. The cells were then transformed with the gapped plasmids (0.1 μ g) using the Ca-MOPS method (Strike et al., 1979). The "survival" of the plasmid was calculated by dividing the number of transformants obtained with a gap lesion plasmid by that obtained with the control gapped plasmid without the lesion. Cultures were grown from individual colonies, and their plasmid contents extracted and subjected to automated DNA sequence analysis, performed by the Biological Services Department in our institute.

Acknowledgments

Z. L. is the Incumbent of The Maxwell Ellis Professorial Chair in Biomedical Research. This research was supported by grants from The US-Israel Binational Science Foundation (#96-00448), the Israel Ministry of Science (#6107), and from The Forchheimer Center for Molecular Genetics.

Received May 7, 1998; revised June 9, 1998.

References

- Boudsocq, F., Campbell, M., Devoret, R., and Bailone, A. (1997). Quantitation of the inhibition of Hfr \times F⁻ recombination by the mutagenesis complex UmuD'C. *J. Mol. Biol.* **270**, 201–211.
- Bridges, B. (1978). DNA polymerase and mutation. *Nature* **275**, 591–592.
- Bridges, B.A., Motershead, R.P., and Sedgwick, S.G. (1976). Mutagenic DNA repair in *E. coli*. III. Requirement for a function of DNA polymerase III in ultraviolet light mutagenesis. *Mol. Gen. Genet.* **144**, 53–58.
- Brotcorne-Lannoye, A., Maenhaut-Michel, G., and Radman, M. (1985). Involvement of DNA polymerase III in UV-induced mutagenesis of bacteriophage λ . *Mol. Gen. Genet.* **199**, 64–69.
- Bruck, I., Woodgate, R., McEntee, K., and Goodman, M.F. (1996). Purification of a soluble UmuD'C complex from *Escherichia coli*. Cooperative binding of UmuD'C to single-stranded DNA. *J. Biol. Chem.* **271**, 10767–10774.
- Burckhardt, S.E., Woodgate, R., Scheuermann, R.H., and Echols, H. (1988). UmuD mutagenesis protein of *Escherichia coli*: overproduction, purification, and cleavage by RecA. *Proc. Natl. Acad. Sci. USA* **85**, 1811–1815.
- Cox, M.M., McEntee, K., and Lehman, I.R. (1981). A simple and rapid procedure for the large scale purification of the recA protein of *Escherichia coli*. *J. Biol. Chem.* **256**, 4676–4678.
- Cull, M.G., and McHenry, C.S. (1995). Purification of *Escherichia coli* DNA polymerase III holoenzyme. *Methods Enzymol.* **262**, 22–35.
- Dutreix, M., Moreau, P.L., Bailone, A., Galibert, F., Battista, J.R., Walker, G.C., and Devoret, R. (1989). New *recA* mutations that dissociate the various RecA protein activities in *Escherichia coli* provide evidence for an additional role for RecA protein in UV mutagenesis. *J. Bacteriol.* **171**, 2415–2423.
- Echols, H. (1981). SOS functions, cancer, and inducible evolution. *Cell* **25**, 1–2.
- Efrati, E., Tocco, G., Eritja, R., Wilson, S.H., and Goodman, M.F. (1997). Abasic translesion synthesis by DNA polymerase β violates the "A-rule." *J. Biol. Chem.* **272**, 2559–2569.

- Eggleston, A.K., and West, S.C. (1996). Exchanging partners: recombination in *E. coli*. *Trends Genet.* **12**, 20–26.
- Friedberg, E.C., Walker, G.C., and Siede, W. (1995). *DNA Repair and Mutagenesis* (Washington, DC: ASM Press).
- Hevroni, D., and Livneh, Z. (1988). Bypass and termination at apurinic sites during replication of single-stranded DNA in vitro: a model for apurinic site mutagenesis. *Proc. Natl. Acad. Sci. USA* **85**, 5046–5050.
- Kato, T., and Shinoura, Y. (1977). Isolation and characterization of mutants of *Escherichia coli* deficient in induction of mutagenesis by ultraviolet light. *Mol. Gen. Genet.* **156**, 121–131.
- Kowalczykowski, S.C., Dixon, D.A., Eggleston, A.K., Lauder, S.D. and Rehrauer, W.M. (1994). Biochemistry of homologous recombination in *Escherichia coli*. *Microbiol. Rev.* **58**, 401–465.
- Kunkel, T. (1984). Mutational specificity of depurination. *Proc. Natl. Acad. Sci. USA* **81**, 1494–1498.
- Kunkel, T.A. (1990). Misalignment-mediated DNA synthesis errors. *Biochemistry* **29**, 8003–8011.
- Lawrence, C.W., Borden, A., Banerjee, S.K., and LeClerc, J.E. (1990). Mutation frequency and spectrum resulting from a single abasic site in a single-stranded vector. *Nucleic Acids Res.* **18**, 2153–2157.
- Livneh, Z. (1986a). Mechanism of replication of ultraviolet-irradiated single-stranded DNA by DNA polymerase III holoenzyme of *Escherichia coli*. Implications for SOS mutagenesis. *J. Biol. Chem.* **261**, 9526–9533.
- Livneh, Z. (1986b). Replication of UV-irradiated single-stranded DNA by DNA polymerase III holoenzyme of *Escherichia coli*: evidence for bypass of pyrimidine photodimers. *Proc. Natl. Acad. Sci. USA* **83**, 4599–4603.
- Livneh, Z., Cohen-Fix, O., Skaliter, R., and Elizur, T. (1993). Replication of damaged DNA and the molecular mechanism of ultraviolet light mutagenesis. *Crit. Rev. Biochem. Mol. Biol.* **28**, 465–513.
- Loeb, L.A., and Preston, B.D. (1986). Mutagenesis by apurinic/aprimidinic sites. *Annu. Rev. Genet.* **20**, 201–230.
- Lohman, T.M., and Overman, L.B. (1985). *J. Biol. Chem.* **260**, 3594–3603.
- Marsh, L., and Walker, G.C. (1985). Cold sensitivity induced by overproduction of UmuDC in *Escherichia coli*. *J. Bacteriol.* **162**, 155–161.
- Mo, J.Y., and Schaaper, R.M. (1996). Fidelity and error specificity of the α catalytic subunit of *Escherichia coli* DNA polymerase III. *J. Biol. Chem.* **271**, 18947–18953.
- Napolitano, R.L., Lambert, I.B., and Fuchs, R.P.P. (1997). SOS factors involved in translesion synthesis. *Proc. Natl. Acad. Sci. USA* **94**, 5733–5738.
- Nelson, J.R., Lawrence, C.W., and Hinkle, D.C. (1996a). Deoxycytidyl transferase activity of yeast *REV1* protein. *Nature* **382**, 729–731.
- Nelson, J.R., Lawrence, C.W., and Hinkle, D.C. (1996b). Thymine-thymine dimer bypass by yeast DNA polymerase ζ . *Science* **272**, 1646–1649.
- Nohmi, T., Battista, J.R., Dodson, L.A., and Walker, G.C. (1988). RecA-mediated cleavage activates UmuD for mutagenesis: mechanistic relationship between transcriptional derepression and post-translational activation. *Proc. Natl. Acad. Sci. USA* **85**, 1816–1820.
- Paz-Elizur, T., Barak, Y., and Livneh, Z. (1997). Anti-mutagenic activity of DNA-damage binding proteins mediated by direct inhibition of translesion replication. *J. Biol. Chem.* **272**, 28906–28911.
- Petit, M.A., Bedale, W., Osipiuk, J., Lu, C., Rajagopalan, M., McInerney, P., Goodman, M.F., and Echols, H. (1994). Sequential folding of UmuC by the Hsp70 and Hsp60 chaperone complexes of *Escherichia coli*. *J. Biol. Chem.* **269**, 23824–23829.
- Radman, M. (1975). SOS repair hypothesis: phenomenology of an inducible DNA repair which is accompanied by mutagenesis. In *Molecular Mechanisms for Repair of DNA*, R.B. Setlow and P.C. Hanawalt, eds. (New York: Academic Press), pp. 355–367.
- Rajagopalan, M., Lu, C., Woodgate, R., O'Donnell, M., Goodman, M., and Echols, M. (1992). Activity of the purified mutagenesis proteins UmuC, UmuD', and RecA in replicative bypass of an abasic site DNA lesion by DNA polymerase III. *Proc. Natl. Acad. Sci. USA* **89**, 10777–10781.
- Roca, A.I., and Cox, M.M. (1990). The RecA protein: structure and function. *Crit. Rev. Biochem. Mol. Biol.* **25**, 415–456.
- Sancar, A. (1994). Mechanisms of DNA repair. *Science* **266**, 1954–1956.
- Sarov-Blat, L., and Livneh, Z. (1998). The mutagenesis protein MucB interacts with single strand DNA binding protein and induces a major conformational change in its complex with single-stranded DNA. *J. Biol. Chem.* **273**, 5520–5527.
- Shinagawa, H., Iwasaki, H., Kato, T., and Nakata, A. (1988). RecA protein-dependent cleavage of UmuD protein and SOS mutagenesis. *Proc. Natl. Acad. Sci. USA* **85**, 1806–1810.
- Sommer, S., Bailone, A., and Devoret, R. (1993). The appearance of the UmuD' C protein complex in *Escherichia coli* switches repair from homologous recombination to SOS mutagenesis. *Mol. Microbiol.* **10**, 963–971.
- Strike, P., Humphreys, G.O., and Roberts, R.J. (1979). Nature of transforming deoxyribonucleic acid in calcium-treated *Escherichia coli*. *J. Bacteriol.* **138**, 1033–1035.
- Sweasy, J.B., Witkin, E.M., Sinha, N., and Roegner-Maniscalco, V. (1990). RecA protein of *Escherichia coli* has a third essential role in SOS mutator activity. *J. Bacteriol.* **172**, 3030–3036.
- Takeshita, M., Chang, C., Johnson, F., Will, S., and Grollman, A.P. (1987). Oligodeoxynucleotides containing synthetic abasic sites. Model substrates for DNA polymerases and apurinic/aprimidinic endonucleases. *J. Biol. Chem.* **262**, 10171–10179.
- Walker, G.C. (1995). SOS-regulated proteins in translesion DNA synthesis and mutagenesis. *Trends Biochem. Sci.* **20**, 416–420.
- Witkin, E.M. (1976). Ultraviolet mutagenesis and inducible DNA repair in *Escherichia coli*. *Bacteriol. Rev.* **40**, 869–907.
- Witkin, E.M. (1991). RecA protein in the SOS response: milestones and mysteries. *Biochimie* **73**, 133–141.
- Woodgate, R., and Levine, A.S. (1996). Damage inducible mutagenesis: recent insights into the activities of the Umu family of proteins. *Cancer Surv.* **28**, 117–140.
- Woodgate, R., Rajagopalan, M., Lu, C., and Echols, H. (1989). UmuC mutagenesis protein of *Escherichia coli*: purification and interaction with UmuD and UmuD'. *Proc. Natl. Acad. Sci. USA* **86**, 7301–7305.