INTRODUCTION

Solar energy is a major source of energy for all living beings. While it sustains most life on earth, it also can cause damage in genetic materials[1]. The spectra of UV wave lengths that reach the surface of the Earth induce crosslinks between adjacent pyrimidines, giving rise to cyclobutane pyrimidine dimers (CPD) and pyrimidine <6-4> pyrimidone photoproducts[2]. These two photoproducts are potent inhibitors of DNA replication and transcription[2,3]. If these photoproducts are left unrepaired, the potential risks that might arise are aberrant gene products from replication error and/or cell death[2]. Because of the harmful effects of photoproducts and the inevitability of sunlight exposure during eons of evolution, cells seem to have developed many mechanisms to repair or tolerate CPDs and <6-4> photoproducts. As evidenced from the existence of multiple repair mechanisms for photoproduct removal in both prokaryotic and eukaryotic cells, nucleotide excision repair (NER) appears to be a predominant as well as a sophisticated repair mechanism- that resolves wide varieties of structurally diverse DNA lesions, including DNA photoproducts[2,4].

In most organisms studied, although CPD is the major UV-induced phototproduct under physiological conditions (the level of CPDs are 10 times higher than that of <6-4> photoproducts), the repair of CPDs in most organisms is far less efficient than the repair of <6-4> photoproducts, as detected by CPD lyases such as T4 endonuclease V (T4 endo V) or CPD-specific antibodies[5-7]. Intriguingly, in human cells more than 80% of excised CPDs have the phosphodiester bond in the CPD interrupted[8,9]. Based on this finding it has been proposed that human cells are able to process CPDs to a pre-excision form[8,9]. Although this concept was proposed two decades ago, enzyme(s) that could convert CPD to CPD* specifically have not been identified, and even the very existence of CPD* in genomic DNA has yet to be demonstrated. Hence, the possibility that the presence of CPD* in excised DNA fragments is the result of DNA degradation has not been excluded.

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Using DNA fragments containing a site-specific CPD*, Zheng *et al.*[10] found that the *E. coli* NER components, UvrA, UvrB and UvrC work in concert to repair CPD* much more efficiently than CPD. In contrast, T4 endo V repairs CPD much more efficiently than CPD*. Together these results suggest that in *E. coli* cells a process may exist to convert CPD to CPD* to facilitate excision repair. These results indicate that photoreactivation (PHR) of CPD* by photolyase and visible light results in a single-stranded DNA break (SSB) that presumably is due to conversion of CPD* to 2 individual pyrimidines and an interrupted phosphodiester bond[10]. Based on these findings, we developed a method to detect CPD* in genomic DNA and, furthermore, using ligation-mediated polymerase chain reaction (LMPCR), we detected CPD* formation at the sequence level. We found that CPD* indeed occurs in wild type cells as well as in *uvrB-* and *uvrC-*, but not *uvrA-*, mutant cells. In searching for the gene products responsible for the conversion of CPD we found that, similar to *uvrA*-mutant cells, *nfi* mutants cells are unable to form CPD* after UV-irradiation. *Nfi* codes for a phosphodiesterase that selectively incises phosphodiester bonds with structural and sequence specificity[11]. We have also confirmed that *uvrA*-mutant cells are more sensitive to UV-induced cell killing than *uvrB-* and *uvrC-* mutant cells, and that both wild-type and *uvrB*-mutant cells can be sensitized to UV-induced killing by introducing the *nfi* mutation. Introducing the *nfi* mutation into wild type and *uvrB*-mutant cells also abolishes their ability to form CPD* in genomic DNA. However, *in vitro* purified UvrA and Nfi, both individually and in combination, cannot convert CPD to CPD*. This conversion requires cell lysates or the addition of *cho* protein, a protein with great homology to the UvrC protein. Together these findings reveal a new mechanism for CPD repair.

RESULTS

1.   CPD* formation occurs in the genomic DNA of UV-irradiated wild type and *uvrB-* and *uvrC-,* but not *uvrA-,* mutant cells

Using synthesized DNA fragments contained either a site-directed CPD* or CPD, Zheng *et al.*, [10] demonstrated that photoreactivation caused site-specific SSB in CPD*, in contrast, photoreactivation of CPD resulted in the monomerization of the dimerized pyrimidine but did not produce SSBs (Fig. 1). These findings provide us with a rationale to develop a method to detect the existence of CPD* in the genomic DNA of UV-irradiated cells, if at all occur. If the formation of CPD* is a cellular process, as it was demonstrated in UV-irradiated human genomic DNA[9], then photoreactivation should produce SSB in the genomic DNA from UV-irradiation. To test this hypothesis, we investigated CPD* formation in the genomic DNA isolated from UV-irradiated NER proficient *E. coli* cells after incubation times of 0, 30, 60, and 90 min. The isolated genomic DNA was photoreactivated with *E. coli* photolyase, denatured by neutral denaturation methods and the resultant DNA was separated by electrophoresis in an agarose gel (as described in Methodology). A typical result is shown in Fig. 2. It demonstrates that photoreactivation induces: 1) no SSB in UV-irradiated genomic DNA isolated from unirradiated cells (lane 4, Fig. 2); 2) no or small amounts of SSB in genomic DNA isolated from cells immediately after UV irradiation (lane 7, Fig. 2); 3) a significant amount of SSB in genomic DNA isolated from UV-irradiated cells after 30 and 60 min of incubation (lanes 10 and 13, Fig. 2); and 4) no SSB in genomic DNA isolated from cells CPD* after 90 min of incubation (lanes 16 and 19, Fig. 2).

We also examined whether CPD* was repaired rather than reversed to CPD using T4 endo V, a glycosylase and an AP endonuclease that specifically incises the phosphodiester bond 5' to a CPD[2]. To determine the incidence of CPDs at different times-- we performed theT4 endo V incision assay with the UV-irradiated cells from the paragraph above. Results in Fig. 2 show that CPDs were induced in cells immediately after UV irradiation (lane 8, Fig. 2), whereas a

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significant amount of CPDs remained after 30 min of incubation (lane 11, Fig. 2), however, CPDs were no longer observed after 60 min of incubation (lanes 14, 17, 20 Fig. 2). Since SSBs neither occurred in the genomic DNA from unirradiated cells after photoreactivation (lane 4 Fig. 2) nor from irradiated cells without photoreactivations at any time points (lanes 6, 9, 12, 15 and 18, Fig. 2), these results suggest that CPD* formation is a cellular event and that CPD* is subjected to repair or reversal (ligation of the interrupted phophodieaster bond in CPD*). Since we have previously shown that CPD* is refractory to T4 endo V activity, these results indicate that CPD* is repaired rather than reversed to CPD[10].

It has been well established that the *uvrA*, *uvrB* and *uvrC* gene products are responsible for the initial recognition and incision of CPD[12,13]. We sought to determine the roles of UvrA, UvrB, and UvrC in CPD* formation, and therefore determined CPD* formation in *uvrA*-, *uvrB*-, and *uvrC*-mutant cells. As expected, DNA isolated immediately after irradiation of cells is susceptible to T4 endo V in all cases (lane 8 in Fig. 3a, 3b and 3c). The amount of CPDs remained stable at all time points in *uvrA* cells (lane 8, 11, 14 and17 in Fig. 3a). UV irradiation results in CPD formation but no CPD*s were observed in *uvrA* cells after photoreactivation even after 90 min of incubation (lane 7, 10, 13 and16 Fig. 3a). These results indicate that the *uvrA* gene product is a putative candidate in CPD* formation.

In *uvrB* and *uvrC* cells, only small amounts of CPD* were detected in genomic DNA immediately after photoreactivaiton (lane 7, Fig. 3b and 3c) but at 30 min incubation a substantial amount of CPD* was formed (lane 10 in Fig. 3b and 3c) and more CPD*s were observed after further incubation (lane 16, 19 in Fig. 3b and 3c). In contrast, CPDs were detected immediately after UV irradiation and the amount of CPDs seem to decrease upon further incubation (lane 8, 11, 14, 17 and 20 in Fig. 3b and 3c). These results indicate that the *uvrB* and *uvrC* gene products are not involved in CPD* formation and hence CPD*s occurred but were not repaired in *uvrB*- and *uvrC*-mutant cells. The observed decreasing levels of CPD and corresponding increasing levels of CPD* formation

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in the course of incubation time are due to conversion of CPD to CPD* in UV-irradiated *uvrB* and *uvrC* cells (lane10 vs. 11, 13 vs. 14, 16 vs. 17 and 19 vs. 20 in Fig. 3b and 3c).

2.   *nfi* mutation abolishes conversion of CPD to CPD*

The results presented above indicate that formation of CPD* is a cellular process and that UvrA, but not UvrB and UvrC, could be involved in it. We have also found that incubation of UV-irradiated genomic DNA with *uvrB-,* or *uvrC-*mutant cell lysates results in CPD* formation with or without addition of purified UvrA protein, whereas purified UvrA alone failed to generate CPD* (data not shown). These results indicate that although UvrA is necessary for the conversion of CPD to CPD* but it is not sufficient and factors other than UvrB and UvrC are needed for this conversion since we have not observed involvement of UvrB or UvrC in CPD* formation. It is well established that UvrA is a DNA-binding protein; it binds to UV-irradiated DNA with a modest affinity4[4,14,15] and the UvrA-CPD complex can attract UvrB binding and possibly also UvrC, to further advance the incision process[4,14,15]. We speculate that binding of UvrA to a CPD may attract a phosphodiesterase, similar to how UvrA-CPD binding recruits UvrB binding[4,14,15]. We also reason that this phosphodiesterase has to function strictly as an endonuclease but not an exonuclease since no SSBs were found in genomic DNA isolated from wild type and *uvrB*-mutant cells unless these DNAs were subjected to PHR. Nfi protein is a likely candidate, since it is known that Nfi is a phosphodiesterase that incises phosphodiester bonds at hypoxanthine and mispaired bases and that Nfi does not function as exonuclease[11]. To test this possibility we examined CPD* formation in *nfi*-mutant cells (MST3*nfi*). Interestingly DNA isolated from irradiated cells is susceptible to T4 endo V in all cases (lane 8, 11,14, 17 and 20 in Fig. 3d) and the amount of CPDs remained stable all across the incubation time points (lane 8, 11, 14, 17 and 20 in Fig. 3d). No CPD*s were observed in UV-irradiated MST3*nfi* genomic DNA even after 90 min of incubation (cf. lane 7, 10, 13, 16 and 19 in Fig. 3d). Similar results were obtained after introduction of *nfi* mutation into

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wild type cells (MST3*nfi*), where no CPD* were observed in UV-irradiated genomic DNA all across the incubation times. However, CPDs were repaired like wild type cells during the incubation times (data not shown). These results indicate that the *nfi* could be a putative agent in the conversion of CPD to CPD*.

3.   Determination of CPD* formation at the sequence level in UV-irradiated wild type *and uvrA-, uvrB-* and *uvrC*-mutant cells

The results above indicate that CPD* formation occurs in the genomic DNA of UV-irradiated wild-type, *uvrB-* and *uvrC*-mutant cells but not in *uvrA-* or *nfi*-mutant backgrounds. We also sought to determine whether the observed sites of CPD* formation are at contiguous pyrimidines and whether the interruption of the phosphodiester bond occurs within the contiguous pyrimidines. It also appears from above results that formation of CPD* is a cellular process and if that is the case then photoreactivation should produce SSB in the genomic DNA from UV-irradiated cells but not in *in vitro* UV-irradiated purified genomic DNA. To test this hypothesis, we investigated CPD* formation at the sequence level under 2 conditions: 1) in the genomic DNA isolated from UV-irradiated *E. coli* cells after different incubation times (0, 5, 15, 60 and 90 min); and 2) in genomic DNA isolated from unirradiated *E. coli* and then subjected to UV irradiation. We have identified sites of CPD* formation at the nucleotide level in *lac Z* promoter region from -80 to -205 in genomic sequence of above-mentioned genetic backgrounds of *E. coli* cells. Genomic DNA isolated from *E. coli* cells with and without UV-irradiation was subjected to PHR, followed by ligation-mediated polymerase chain reaction (LMPCR) and the resultant DNA was separated by DNA sequencing gel electrophoresis. A typical result of CPD* formation in the *lac Z* promoter region in wild type *E. coli* cells is shown in Fig. 4 (lanes 3-10). The results show two distinct features: 1) PHR induces bands only in UV-irradiated cells (cf. lane 3 to lanes 5 to 9), and PHR does not result in bands in UV-irradiated genomic DNA (lane 4); and 2) both intensity and number of sites of CPD* formation are functions of postirradiation incubation (Fig. 4 lanes 5 to 10). Of the23 bands found after PHR (PHR bands)—22 can be attributed, with

certainty to regions with contiguous pyrimidines (pyrimidine clusters). Two clusters of PHR bands can be attributed to –TTT- and –TTTT- regions, although, these two bands are at slightly higher positions than expected. One cluster PHR band (identified with a ? mark) occurs at a –CGCGCG- sequence. Intriguingly, we have found that T4 endo V treatment also induces bands in this sequence (lanes 3 and 14 in Fig. 5). Our result shows that PHR allows detection of SSB in most if not all of these pyrimidine clustered regions (≥ 2 pyrimidines) in UV-irradiated cells after different incubation times. Since PHR bands (CPD*) are neither observed in unirradiated control DNA (lane 3 in Fig. 4) nor in control DNA subjected to UV-irradiation *in vitro* (lane 4 in Fig. 4) we interpret that these bands occur as a result of CPD* formation in these sequences and that the formation of CPD*s is a result of cellular processes. The results also show that the formation of CPD* in wild-type cells is a function of time. The amount of CPD*s increases from 0 min to 15 min (lanes 5-7, Fig. 4) and then decreases with further incubation (lanes 8-10, Fig. 4), an indication that CPD*s are being repaired. Formation of CPD*s appears to be a rapid process, with a significant amount of CPD*s being formed immediately after UV irradiation, although it should be noted that it takes 7 min to harvest the irradiated cells by centrifugation before the cells are lysed. It is worth noting that LMPCR/DNA sequencing gel electrophoreses method is much more sensitive for detecting SSB than the agarose electrophoresis method used in Fig. 2 and 3. Intriguingly no CPD or CPD* formation was detected in the pyrimidine tract of a 5'–TTTTTCTTTTC- sequence (Please see lanes 3 and 14 in Figs. 3, 5). One possible explanation is that a triplex is formed in this region through Watson and Crick and Hogsten base pairing and, as Tang *et al.*, [16] demonstrated, triplex formation inhibits CPD formation.

As expected, CPD* formation was not observed in the genomic DNA of irradiated cells after introducing *nfi*-mutation in wild type strain (MST1*nfi*, lanes 13-15, Fig. 4). Although there are 23 CPD* clustered regions detected in the sequence shown in MST1 cells (Fig. 4a), in contrast, 22 of these CPD* clusters totally disappeared in the same sequence in MST1*nfi* cells (Fig. 4b). One CPD

sequence (TC, at bottom of the gel) seems likely to convert to CPD* in MST1*nif* cells, albeit in a very low intensity (lanes 13, 15; Fig. 4b). These results clearly indicate that Nfi is certainly a candidate in the conversion of CPD to CPD* and perhaps Nfi incises phosphodiester bonds between contiguous pyrimidine bases as it has been defined as an endonuclease and a phosphodiesterase[11].

In UV-irradiated *uvrB*-mutant cells (MST3), CPD*s form at the same sequences as it demonstrated in wild type cells. However, since unlike in wild type cells, no reduction or repair of CPD*s was observed after 60 min incubation, it appears that the accumulation of CPD* is a function of post-irradiation incubation time (lanes 6-8; Fig. 5). These results indicate that *uvrB*-mutant cells are deficient in CPD* repair. On the contrary, introducing *nif* mutation into *uvrB*-mutant cells fully abolished CPD* formation in postirradiated MST3*nfi* cells (lanes 11-13, Fig. 5) as was observed in MST1*nfi* (Fig. 4b). These results further validate involvement of Nfi in CPD* formation.

In UV-irradiated *uvrA* cells, as expected, no CPD* formation was observed all across the incubation time points (lanes 5-7 in Fig. 6). Since UV irradiation produces T4 endo V sensitive sites (CPDs) whereas CPDs remain unchanged at different incubation times, these results indicate that no conversion of CPD to CPD* occurs in *uvrA*-mutant cells and *uvrA*-mutant cells are deficient in CPD repair. In UV-irradiated *uvrC* cells (MST8), CPD* forms at the sequences (lanes 10-12 in Fig. 6) that are the same as in wild type (Fig. 4a) and in *uvrB*-mutant cells (lanes 6-8; Fig. 5). The accumulation of CPD* formation occurs as function of time as it was demonstrated for *uvrB*- mutant cells, indicating that *uvrC* cells are deficient in CPD* repair (lanes 10-12 in Fig. 6).

To show that the CPD* formation demonstrated above in *lac Z* promoter sequences in *E. coli* was not any artifact of a particular gene or a particular DNA strand, we examined CPD* formation in *lac I* non-transcribed (sequences 104 to 242) and transcribed (sequences 275 to 140) strands in wild type *E. coli* cells. Similar results were obtained in the *lac I* coding region (data not shown) as is described for *lac Z* noncoding (promoter) sequences (lanes 3-10, Fig. 4).

4. Conversion of CPD to CPD* requires the UvrA, Nfi, and Cho proteins

In vivo results indicate that conversion of CPD to CPD* is a cellular process that requires the UvrA and Nfi, but not the UvrB and UvrC, proteins. To investigate whether UvrA and Nfi are sufficient for this process and directly involved in this conversion, we examined CPD to CPD* conversion *in vitro* in UV-irradiated genomic DNA with and without treatment with purified UvrA and Nfi. Results in Fig. 7 (lanes 6, 8, 9, 11, and 13) show that no conversion of CPD to CPD* occurs in UV-irradiated genomic DNA treated with UvrA and Nfi; this result indicates that UvrA and Nfi are not sufficient to convert CPD to CPD* and that factor(s) other than UvrA and Nfi are required in this process.

To search for other candidate(s) that might be involved in processing CPD, we focused our attention to UvrA protein-dependent endonucleases and DNA-binding proteins since the UvrA protein is required for CPD to CPD* conversion process. In NER incision, UvrA first binds to the photoproducts and the UvrA-photoproduct complex consequently attracts UvrB and UvrC proteins to execute the dual incision[4,14,15]. It is unlikely that UvrA, UvrB and UvrC work in concert to convert CPDs to CPD*s since CPD* are not observed in UV-irradiated *uvrB*- and *uvrC*-mutant cells. UvrD has been shown to facilitate UvrABC-dependent excison repair synthesis, however, UvrD functions as a helicase and no endonuclease or exonuclease activity has thus far been associated with this protein[17,18]. Recently it has been reported that a SOS-inducible *ydjQ* gene product functions as a repair enzyme in a UvrA- and UvrB-dependent manner[19]. This protein is called Cho (a *uvrC* homolog) that shares significant homology with N-terminal half of UvrC, and in conjunction with UvrA and UvrB, can incise 3' to a cholesterol-DNA damage[19]. While *cho*-defective mutant cells are not more UV-sensitive than wild-type cells, *uvrC-cho*–double mutant cells are more UV sensitive than *uvrC*-mutant cells[19]. It is possible that the UvrC and Cho proteins have an independent function in the repair of CPD. Since we found that UvrC is not needed in processing CPDs to CPD*s, we therefore determined the role of Cho proteins in this process. Results in Fig. 7 (lane 7 vs. lanes 10, 12 and 14) show that while Cho alone is unable to process CPD, in combination with UvrA

and Nfi,-- it can convert CPDs to CPD*s. Together these results indicate that UvrA, Nfi and Cho are necessary and sufficient to convert CPDs to CPD*s.

5. *Nfi* mutation sensitizes UV-induced cell killing in wild type and *uvrB* cells

It is well established that excision of CPDs photoproducts requires that UvrA, UvrB and UvrC proteins work in concert, and mutation in any of these 3 genes causes cells to be equally deficient to CPD removal. Intriguingly, *uvrA*-mutant cells are significantly higher sensitive to UV-induced cell killing than *uvrB*- and *uvrC*-mutant cells[20]. Our findings that both *uvrB*- and *uvrC*- but not *uvrA*-mutant cells are able to convert CPDs to CPD*s and that an additional *nfi* mutation abolishes CPD* conversion in wild-type (data not shown) and *uvrB*-mutant cells suggest that failure to convert CPDs to CPD*s in *uvrA*-mutant cells is why *uvrA*-mutant cells are more sensitive to UV-induced cell killing than *uvrB*-mutant cells. To test this possibility we determined the effects of a *nfi* mutation in UV-induced cell killing by determining UV survival curves in *nfi*-, *uvrB*-, *uvrBnfi*-, and *uvrA*-mutant cells as well as wild-type *E. coli*. Results in Fig. 8 show that the *nfi* mutation sensitizes UV-induced cell killing effect not only in wild- type but also in *uvrB*-mutant *E. coli* cells. Furthermore, the UV sensitivity of *uvrBnfi*– double mutant cells is similar, if not identical, to that of *uvrA*-mutant cells. These results indicate that the conversion of CPDs to CPD*s is a repair process and that failure to convert CPDs to CPD*s could be the major reason why *uvrA*-mutant cells are more sensitive to UV irradiation than *uvrB*- or *uvrC*-mutant cells.

6.   CPD*, but not CPD, allows DNA polymerases 1-mediated translesion synthesis

It is well established that the majority of the UV-induced cytoxicity can be attributed to CPDs blocking DNA replication and transcription[2]. The increased survival of CPD* proficient cells (Fig. 8) was really intriguing and it raises the possibility that CPD*s may allow translesion synthesis and consequently reduces UV-induced lethality. To test this possibility we have constructed fragments

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containing a site-directed CPD* (as described in Materials and Methods) and used these constructs as templates for DNA polymerase 1– mediated translesion synthesis. Results in Fig. 9 show that CPD*s allow efficient translesion synthesis (Fig. 9b). Furthermore, sequencing the bypassed DNA fragments shows that 2 dATPs are inserted opposite to the CPD* template (Fig. 9c), indicating that CPD*s are instructive templates for DNA replication. Thus the different UV sensitivities between *uvrB-* and *uvrA*-mutant cells is due to *uvrA*-mutant cells being unable to convert CPDs to CPD*s, and would indicate that unrepaired CPD*s may not be as genotoxic as CPDs.

DISCUSSION

There has been considerable confusion about the differential incision of CPD between *in vitro* and *in vivo*. In this study, we described why this has been so and elucidated a new CPD preincision processing mechanism in NER in *E. coli*, and genes involved in this process. UvrABC nucleases recognize and repair a wide range of substrates and DNA lesions. It is difficult to see why a CPD is a poorer substrate than a <6-4> photoproduct when more than 80% of UVC-induced photoproducts are CPDs[5]. In addition to UV-photoproducts, UvrABC nucleases repair DNA bulky adducts regardless of whether the damage causes helix destabilization or stabilization[4,21]. The bulky DNA damages such as those induced by benzo(a)pyrene diol epoxide and N-acetoxy-2-acetyl-aminofluorene, are efficiently repaired by the UvrABC nuclease, whereas CPD is indeed a poor substrate for purified UvrABC nuclease[4,22]. Nevertheless, CPD is repaired very efficiently and effectively in *E. coli* cells[23]. The *E. coli* photolyase has been shown to enhance CPD removal by UvrABC nucleases *in vitro*[24]. The possible explanation for this mechanism is that photolyase accelerated CPD recognition and UvrABC complex turnover which otherwise remained tightly bound to CPD-containing double-stranded DNA. However, this phenomenon was true only for an *in vitro* reaction as a photolyse deficient strain was no more UV-sensitive than wild type background[24].

In this study we demonstrated CPD* formed in wild-type as well as in *uvrB* and *uvrC* mutant cells whereas CPD* was neither occurred in *uvrA* and *nfi* mutant cells, and nor in *cho* mutant cells (data not shown). These results indicate that UvrA, Nfi and Cho are the putative candidates that participate in a preincision processing of CPD to CPD* in *E. coli* cells. It is difficult to vision why a preincision step of CPD be facilitating CPD removal in cells where UvrABC nucleases are sufficient to incise CPD *in vitro*, albeit in a very disproportionate rate compared to *in vivo*. Previously, Zheng *et al.*, [10] demonstrated that CPD* is a much better substrate than a CPD for UvrABC excision. In human cells more than 80% of excised CPDs have been found to be in the CPD* form[8,9]. It is worth mentioning that although CPD is repaired efficiently *in vivo* in human cells,

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paradoxically, purified NER components from human cells barely repair CPD *in vitro*[25]. It seems more likely that CPD* is a preincision form of CPD that facilitates excision in human cells and it is reasonable to propose, similar to humans, that in the *E. coli* cell system CPD*s are a much better substrate than CPDs. In the *E. coli* NER system, CPD removal through dual incision activity is carried out by sequential function of *uvrA*, *uvrB* and *uvrC* gene products and mutation of any of these genes are equally deficient in CPD removal. Interestingly, Tang and Ross[20] observed that *uvrA* mutant cells are hypersensitive to UVC-mediated cell killing compared to *uvrB*- and *uvrC*-mutant cells, indicating that UvrA may be able to process a sub-group of photoproducts independent of UvrB and UvrC. However, in CPD repair UvrA recognizes the damage site and employs UvrB to bind to the damaged site[12], and no repair function of UvrA independent of UvrB and UvrC has been reported to date. Thus, it is reasonable to assume that UV-hypersensitivity of *uvrA* mutant cells compare to *uvrB* or *uvrC* mutant cells is because *uvrA*-mutant cells are unable to convert CPDs to CPD*s, implying that unrepaired CPD*s are not necessarily as genotoxic as CPDs. Another line of support for this hypothesis arises from the *nfi* mutation enhanced UV-induced killing of *uvrB*-mutant and wild-type cells, since both are unable to convert CPD to CPD*. *In vitro* DNA polymerase 1– mediated translesion bypass of CPD*, but not CPD, sheds more light on why CPD* generating cells gain better survival against UV-induced cell lethality. Perhaps, conversion of CPD to CPD* reduces the DNA structural constraint caused by CPD that avoids replication arrest and cell death at the cost of elevated mutagenesis.

The excision repair in *E. coli* is carried out by sequential and partially overlapping functions of UvrA, UvrB and UvrC proteins. UvrA recognizes the damage and serves as matchmaker for UvrB to bind to the damaged site, and UvrC subsequently joins to exert dual incisions[12-14]. In *E. coli* and some other bacteria, Cho acts in conjunction with UvrA and UvrB, and makes 3' incision to 3' end of the lesion[19]. Working coordinately with UvrC, Cho makes some contribution to UvrABC excinuclease DNA repair[19]. In a reconstituted *in vitro*

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system, we found that purified UvrA, Cho and Nfi are necessary to convert CPD to CPD*. In addition, we found that Nfi does not bind to UV-irradiated DNA (data not shown), suggesting that UvrA and Cho proteins may function as matchmakers for Nfi-CPD binding, which consequently results in the conversion of CPD to CPD*. Taking together the *in vivo* and *in vitro* results, we propose that conversion of CPDs to CPD*s by UvrA, working in concert with the Nfi and Cho proteins, is a preincision processing step that facilitates CPD repair in *E. coli* cells as shown below

$$(UvrA)_2 + CPD \Longrightarrow (UvrA)_2. CPD \xrightarrow{Cho} (UvrA)_2. Cho. CPD \xrightarrow{Nfi} $$

$$(UvrA)_2. Cho. Nfi. CPD \Longrightarrow (UvrA)_2. CPD \xrightarrow{UvrB} UvrB\ CPD^* \xrightarrow{UvrC} $$

Cho + Nfi + UvrA

$$UvrB. UvrC. CPD^* \Longrightarrow CPD^*\ Excision$$

In summary, we found that CPD*s are a preincised state of CPDs which occurs as a cellular mechanism in wild type as well as in *uvrB-* and *uvrC*-mutant *E. coli* cells but not in *uvrA-*, *cho-* and *nfi*-mutant cells. *nfi* mutation enhances UV-induced cell killing in both wild type and *uvrB*-mutant cells. In an *in vitro* reconstituted system UvrA, Cho and Nfi proteins are able to convert CPDs to CPD*s and CPD*s allow efficient and accurate translesion DNA synthesis. It is possible that conversion of CPDs to CPD*s may release CPD-induced DNA constraint, thereby allowing efficient DNA wrapping around UvrB and facilitating UvrABC incision. We have also demonstrated that the Mfd transcription-repair coupling factor has a significant role in CPD* induction and repair (another

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manuscript in preparation). This subpathway is likely responsible for a subset of CPDs that are either insensitive to UvrABC and/or required to be repaired promptly to coordinate with other physiological processes such as transcription and/or DNA replication. Finally, this work has opened up a new avenue of research in NER that has been remained as long standing biological dilemma for decades in both prokaryotes and eukaryotes.

Fig. Legends

Figure 1 Structures of CPD and CPD* and the resultant products following photoreactivation.

Figure 2 The sensitivity of T4 endo V and photoreactivation of the genomic DNA isolated from UV-irradiated wild-type *E. coli* cells at different times of post-irradiation incubation. Cells were irradiated with 25 J/m^2 of 254-nm UV light and incubated in growth medium for 0-180 min. The genomic DNA was isolated, subjected to T4 endo V or photolyase treatments, denatured, and the resultant DNAs were separated by 0.5 % agarose gel electrophoresis, as described in Materials and Methods. Lanes 1-2, HindIII- digested λ DNA with and without formamide denaturation. Cont DNA: genomic DNA isolated from control cells, lanes 3-5. Lanes 6-20, DNA isolated from UV-irradiated cells at different incubation times (0, 30, 60, 90, and 180 min). In lanes 5, 8, 11, 14, 17 and 20, DNA was first photoreactivated, the photoreaction enzyme was then removed and then treated with T4 endo V. Symbols: PHR, DNA subjected to photoreactivation treatment; T4 endo V, DNA subjected to T4 endo V treatment.

Figure 3 T4 endo V and photoreactivation sensitivities of genomic DNA isolated from UV-irradiated (a) *uvrA-*, (b) *uvrB-*, (c) *uvrC-* and (d) *nfi-*mutant *E. coli* cells at different post-irradiation incubation times (0, 30, 60, 90, and 180 min). Cell growth, UV irradiation, genomic DNA isolation, T4 endo V and photoreactivation treatments, DNA separation methods are the same as in Fig. 2.

Figure 4 Time course of CPD* sites at the sequence level in the *lac Z* gene in post-irradiated wild type (MST1) and *nfi* mutant *E. coli* cells (MST1*nfi*). The same genomic DNA as described in Fig. 2 was subjected to photoreactivation treatment and ligation-mediated PCR and the resultant DNA was separated by electrophoresis in an 8% denaturing sequencing gel, transferred to nitrocellulose

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membrane by electroblotting and hybridized with ^{32}P-labeled probes. Lanes 1 and 2 are A+G and C+T Maxim-Gilbert sequencing reactions[25]; Lanes 3-10 are DNA from wild- type *E. coli* cells; Lanes 11-15 are DNA from *nfi* cells. Symbols: Cont, genomic DNA isolated from control cells (lane 3); Cont + UV, control DNA irradiated with UV (25 J/m^2) (lane 4); 0, 5, 15, 30 and 60, DNA isolated from UV (25 J/m^2) irradiated cells at different incubation times (min). Contiguous pyrimidines sites (left) and the corresponding CPD* formation sites (right) are indicated.

Figure 5 Time course of CPD* sites at the sequence level in the *lac Z* gene in post-irradiated *uvrB-* (MST3) and *uvrBnfi-* (MST3*nfi*) cells. The same genomic DNA as described in Fig. 3b was subjected to the same treatment as described in Fig. 4. Lanes 1 and 2 are A+G and C+T Maxim-Gilbert sequencing reactions (Maxam and Gilbert, 1980); lanes 3-8 are DNA from *uvrB-* mutant cells; lanes 9-14 are DNA from *uvrBnfi-* mutant cells; lane 3, DNA isolated from UV-irradiated *uvrB* cells at time 0 and subjected to T4 endo V treatment; lane 14, DNA isolated from UV-irradiated *uvrBnfi* cells at time 0 and subjected to T4 endo V treatment. Symbols: Cont, control DNA (lane 4); Cont + UV, UV-irradiated control DNA (lane 4); 0, 15 and 60, DNA isolated from UV irradiated cells at different incubation times (min). CPD* formation sites (right) are indicated.

Figure 6 Time course of CPD* sites at the sequence level in the *lac Z* gene in post-irradiated *uvrA-* (MST13) and *uvrC*-mutant *E. coli* cells (MST8). The same genomic DNA as described in Fig. 3 was subjected to the same treatment as described in Fig. 4. Lanes 1 and 2 are A+G and C+T Maxim-Gilbert sequencing reactions[26] (Maxam and Gilbert, 1980); lanes 2-7 are DNA from *uvrA-* mutant cells. Lanes 8-12 are DNA from *uvrC*-mutant cells. Lane 2 is genomic DNA isolated from time 0 and subjected to T4 endo V treatment. Symbols: Cont, control DNA (lane 3); Cont + UV, UV-irradiated control DNA (lanes 4); 0, 15 and 60, DNA isolated from UV irradiated cells at different incubation times (min). CPD* formation sites (right) are indicated.

Figure 7   *In vitro* regeneration of CPD* in the *lacZ* promoter sequences by purified UvrA, Nfi and Cho proteins. Genomic DNA isolated from *E. coli* cells were irradiated with UV (60 J/m^2) and used as substrates. Purified UvrA (15 nM), Nfi (15 nM) and Cho (25 nM) were added to the DNA substrates individually, pairwise or all together in UvrABC reaction buffer and the mixtures were incubated at 37 ^{o}C for 60 min. After incubation DNAs were purified and then subjected to photoreactivation and ligation-mediated PCR in the same manner as described in Fig. 4 - 6. The amount of DNA in the gel is the same for lanes 3 to 8, and lanes 11 and 12. The amounts of DNA in lanes 9 and 10, and in 13 and 14 are 0.5- and 2-fold of the amount DNA in other lanes, respectively. CPD* formation sites (right) are indicated.

Figure 8   UV sensitivities of *E. coli* wild type cells (MST1) and *uvrA-* (MST13), *uvrB-* (MST3), *nfi-* (MST1*nfi*) and *uvrBnfi–* (MST3*nfi*) mutant *E. coli* cells. Cells were grown in LB to late log phase, harvested by centrifugation, resuspended in M9 buffer, irradiated with different doses of UV (0, 5, 10 and 20 J/m^2) and then plated on LB plates. The colonies were counted after 24 h incubation at 37 ^{o}C. All the experiments were performed under yellow light. The results represent 3 independent experiments. The lengths of the bars represent the ranges of the numbers.

Figure 9   Translesion synthesis using templates containing a site-specific CPD*. The template construction and the DNA synthesis mediated by the DNA polymerase Klenow fragment (exo$^-$) are described in the Materials and Methods. (a) Schematic presentation of *in vitro* DNA synthesis using templates containing a site-specific CPD* or CPD. (b) Electrophoretic separation of DNA synthesis products. (c) DNA sequencing results of bypass products. Noted A bases are incorporated into the positions of the synthesized DNA strand that are opposite to CPD* (T-T*) positions (T$_{28}$ and T$_{29}$) in the template strand.

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Materials and Methods

Bacterial strains:

      E. coli strains used in this study are listed in Table 1. MST1 (HF4714) is an *E. coli* K-12 and *E. coli* C hybrid strain; its isogenic derivative strains MST3 (*uvrB5*), MST8 (*uvrC34*) and MST13 (*uvrA6*) are constructed by P1 and T4 transduction[27]. The *nfi* mutation was introduced into MST1 and MST3 strains by P1 transduction[28].

Strains	Repair associated marker	Genotype	Reference or Source
MST1	Wt	*thr-1 leu-6 his-4 argE3* or *arg-49 lacY1 galK2 rpsL31* or *rpsL154 supE44*; an *E. coli* K-12 x *E. coli* C hybrid; φXsensitive	HF4714; T. Kunkel
MST3	*uvrB5*	same as MST1 except Gal$^+$	Tang *et al.*, 1982[27]
MST8	*uvrC34*	same as MST1 except His$^+$	Tang *et al.*, 1982[27]
MST13	*uvrA6*	same as MST1 except Arg$^+$	Tang *et al.*, 1982[27]
MST1::*nfi*	*nfi::cat*	same as MST1 except Cat$^+$	This work
MST1::nfi	*Nfi::cat*	Same as MST3 except Cat*	This work
BW1160	*Nfi::cat*	recD1903::mini-Tn10	Guo and Weiss, 1998[29]

Bacterial growth, UV irradiation and colony forming ability determination

      The bacterial cells were grown in LB (Luria broth; 10 g bacto-tryptone, 10 g NaCl, and 5 g yeast extract in 1 lit distilled water) to stationary phase at 37 ^{o}C and then diluted into fresh LB (1:500) and further grown to A_{600} = 0.5. Cells were centrifuged (5,000 rpm), washed, suspended in M9 buffer (6 g Na_2HPO_4, 3g KH_2PO_4, 5 g NaCl, 1g NH_4Cl, 0.12 g $MgSO_4$, and 0.011 g $CaCl_2$ in 1 liter distilled water), and UV irradiated (germicidal lamps emitting principally 254 nm) with different fluences. After UV irradiation, cells were diluted in M9 buffer and plated

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in LB plates (LB with 12 g of Bacto-agar [Difco Laboratiries] per liter). The plates were incubated at 37 ^{o}C overnight and the colonies were counted.

UV irradiation and genomic DNA isolation:

Stationary phase culture was diluted 1:20 (A_{600} = 0.1) in LB and grown at 37°C to A_{600} = 0.3-0.4. Cells were centrifuged, washed with M9 buffer, and resuspended in the original volume of M9 medium. An aliquot of 30 ml of the suspension was spread on 100 mm petri-dish and, while shaking, UV irradiated at a fluence of 25 J/m^2 (fluence rate, 1 J/m^2 per sec, measured with UVX digital radiometer). Cells were either harvested immediately by centrifugation or supplemented with equal volume 2 fold concentrated LB (pre-warmed to 37 ^{0}C) and incubated at 37 ^{0}C for varying lengths of time followed by centrifugation and harvesting for genomic DNA isolation. The bacterial cell pellet was resuspended in 1 ml Tris buffer with lysozyme (50 mM Tris-HCl, pH 8, lysozyme 1 mg/ml and 25% sucrose) and lysed by incubation with 2 ml of lysis buffer (10 mM Tris-HCl, pH 8, 10 mM EDTA, 10 mM NaCl, 0.5% SDS, 100 mg/ml proteinase K) at room temperature (RT) for 10 min. DNA was purified by multiple phenol-ether extractions, ethanol precipitation, and finally dissolved in TE (10 mM Tris, pH 8.0, 1 mM EDTA). For *in* vitro treatment, purified genomic DNA from unirradiated cells in small droplets on Parafilm was exposed to UV under the same conditions. All manipulations, from UV irradiation to phenol-ether extractions, were carried out under yellow light.

UvrA, T4 endo V, photolyase, Nfi and Cho protein preparations

UvrA proteins were prepared as previously described[22]. T4 endo V proteins were kindly provided by Dr. Steven Lloyd, Oregon State University. Photolyases were prepared according to the method described by Sancar and Sancar[30]. *E. coli* Nfi proteins were purified as a C-terminal hexa-histidine tagged protein as previously described[11]. Although we have prepared both Cho and Cho-Nfi fusion proteins, we have found that our Cho protein preparations contain nonspecific nucleases. In contrast, no nonspecific nuclease activity was found in

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our Cho-Nfi fusion proteins. Furthermore, we found that Cho-Nfi fusion proteins have Nfi activity the same as Nfi protein alone. Therefore, we used Cho-Nfi fusion proteins to check for Cho activity. To prepare Cho-Nfi proteins, CHO-Nfi fused gene fragment were prepared by PCR using two sets of primers. Primer set 1 (TATGGTGGTACGGCGTTTAACTTCTCCGCG, Cho forward; AGCGCGTAATGACGCGAGATCACTGGCTCGCTGGTCATTCGCCGGATC, Cho reverse) will generate an 896 nucleotides *cho* gene fragment containing 21 nucleotides derived from the N-terminal sequence of *nfi* gene (underlined). Primer set 2 (GATCTCGCGTCATTACGCGCTCAACAAATC, Nfi forward; GGGGCTGATTTGCTGTATAGCGCAC GAA, Nfi reverse) will generate the *nfi* gene fragment. The *cho* and *nfi* PCR gene products were then combined, denatured, hybridized and PCR were performed with PDT1 primers (Cho forward and Nfi reverse) to generate the fused *cho-nfi* gene fragment, PDT1(1542 nucleotides). PDT1 PCR product was purified and a second PCR reaction was performed, using PDT1 as the template and PDT2 primers (TGGTGGTACGGCGTTTAACTTCTCCGCG, PCR2 forward; TCGAGGGGCTGATTTGCTGTATAGCGCACGAA, PCR2 reverse) to generate PCR product PDT2. Equal amount of PDT1 and PDT2 were mixed, denatured by heating to 90 ^{0}C for 10 min, and then renatured slowly by cooling over a 2 h period to RT. The renatured product, 25% of which has the ready to ligate NdeI and XhoI site at the N-and C-terminus, respectively, was then ligated into pET22b- plasmid that was precut with Nde1 and Xho1 restriction enzymes. The ligated plasmid was then used to transform XL1-blue *E. coli* cells. pET22b-Cho-Nfi plasmid containing the fusion gene is then purified from XL1-blue cells, and used to transform BL21(DE3) *E. coli* host to generate the Cho-Nfi overproducing *E. coli* strain. Overexpression of Cho-Nfi fusion protein achieved by IPTG induction and the fusion protein was purified using Fe-chelate column as previously described for Nfi protein[11].

CPD and CPD* determinations at the genomic level

CPD* detection was based on our previously finding that photoreactivation (PHR) of CPD* results in SSB at the CPD* site (Fig. 1). This finding provides us with a tool, using PHR-induced SSB production, for detection of CPD* formation in *E. coli* genomic DNA[10]. PHR was carried out by irradiating the genomic DNA (2 μg) in 100 μl reaction mixture (5 mM Tris, pH 7.7, 1 mM EDTA, 100 mM NaCl, 10 mM DTT and 0.03 mM photolyase), with two fluorescent lights (F15T8 CW 15W, General Electric) for 60 min at 5 cm distance. CPD formation was detected by sensitivity to T4 endo V. The reactions were carried out in a reaction mixture (50 μl) containing, genomic DNA (2 μg), 50 mM Tris-HCl (pH 7.7), 100 mM NaCl, 1 mM EDTA and 6.5 nM T4 endo V. The reaction mixture was incubated at 37 °C for 1 h. Both CPD and CPD* reactions was then stopped by multiple phenol-ether extractions and the DNAs were ethanol precipitated, resuspended in TE buffer, denatured by 90% formamide (37 °C, 60 min), and separated by electrophoresis in an agarose gel (0.5%) in TBE buffer[22].

Detection of CPD* at the sequence level by ligation-mediated PCR

We used LMPCR method to determine that the PHR-induced SSB has indeed occurred at CPD* site in *lacZ*, and *LacI* gene sequences. LMPCR method is the same as previously described[31]. To analyze the *lacZ* region, a set of three primers were designed at the operator region for the nontranscribed strand. The sequences of the primers are following primers were used: primer-1, 5'-GGGCAAACCAGCGTGGAC; primer-2, 5'GACCGCTTGCTGCAACTCTCTCAG; primer-3, 5'-GCAACTCTCTCAGGGCCAGGCGGTG. An aliquot of 2-5 μg of treated DNA (+ or - PHR) was subjected to primer extension (primer 1), ligation with universal linkers, then PCR amplification (primer 1 and primer 2, 21 cycles). T4 DNA polymerase (New England Biolab) and Vent DNA polymerase (New England Biolab) were routinely used for primer extension and amplification reactions, respectively. The amplified fragments were separated on 8% urea-polyacrylamide denatured sequencing gel, electroblotted on a nylon membrane and hybridzed with ^{32}P labeled gene specific single stranded probes (synthesized

by using genomic DNA as template and primer 3 for asymmetric PCR) and detected by exposing the membrane to a Cyclone PhosphorImager (Packard, Meriden, CT) first and then to an X-ray film (Kodak).

Conversion of CPD to CPD* *in vitro* by UvrA, Nfi, and Cho proteins

To determine the role of *uvrA, nfi* and *cho* gene products in CPD to CPD* conversion, genomic DNA (2 μg), isolated from unirradiated MST1 cells, was irradiated with UV (60 J/m^2), and then reacted with purified UvrA (15 nM), Nfi (15 nM) and Cho (25 nM), singly, pair wise and in combination in UvrABC reaction buffer at 37 ^{o}C 60 min. At the end of incubation the proteins were removed by multiple phenol/ether extractions, DNA was further purified by ethanol precipitation and finally redissolved in TE buffer. CPD* formation in the *lacZ* gene sequence of these DNAs was then detected by PHR reaction and mapped by the LMPCR method as previously described.

Construction of a DNA fragment containing a site-specific CPD*

A 41-mer (5'-CAGTGATGAGAGACGTGCGTACACGAGTAGTGCGTGACAAT-) and a 24-mer (TGGTGTAACGCAGTGATACAGTG-) were chemically synthesized (Midland Co. TX). To ensure the formation of CPD* lesion at a single specific site, these oligomers were designed to contain no adjacent pyrimidines. The 3' - end T of the 41-mer and the 5'- end T (phosphorylated and labeled with ^{32}P) of the 24-mer, aligned together by a complementary 46-mer splint, were joined through the formation of a CPD* lesion by UV irradiation (4000 J/m^2, 254 nm). The resulting CPD* containing 65-mer was separated on a 13% polyacrylamide-8M urea gel.

In vitro DNA replication system and identification of the sequence of translesion synthesis products

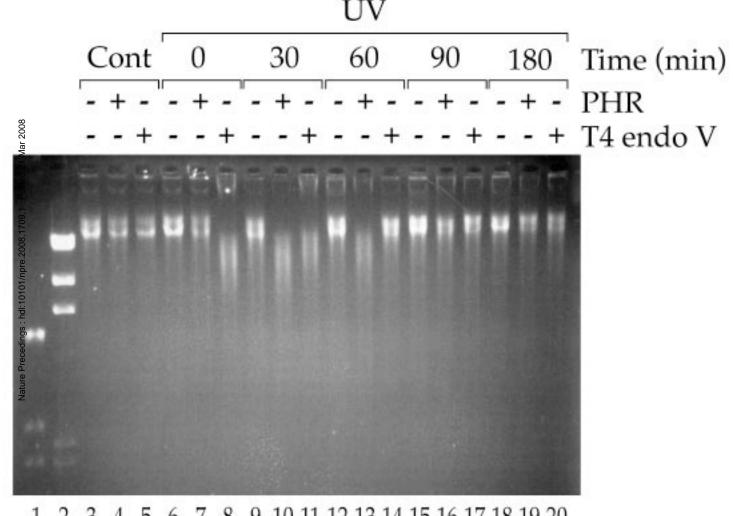The system consists of a primed-template obtained by hybridizing a 21-mer to a 65-mer which contains a site-specific CPD* constructed as described above. This system represents a "running start" for CPD* in DNA replication synthesis

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and occurs prior to the polymerase encounters CPD*. The primed-template (0.1 pmol) was incubated at 37 ^{0}C for 30 min with 0.2 unit of the *E. coli* DNA polymerase (exo$^-$ Klenow fragment) in the presence of 5 mM Mg++ and varying concentrations of dNTP. The products were separated on a 16% polyacrylamide-8M urea sequencing gel, product bands excised and sequenced by Sanger method[32].
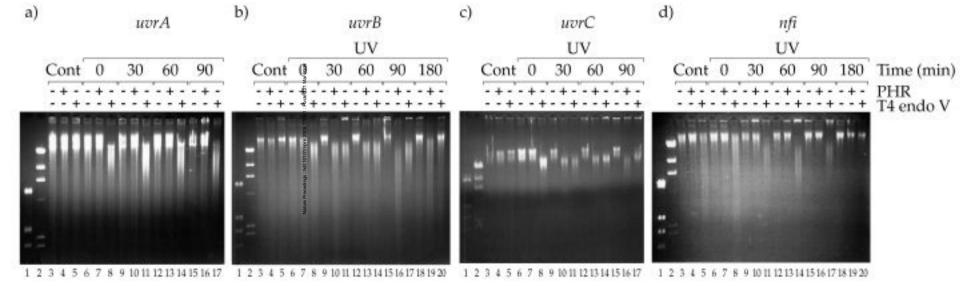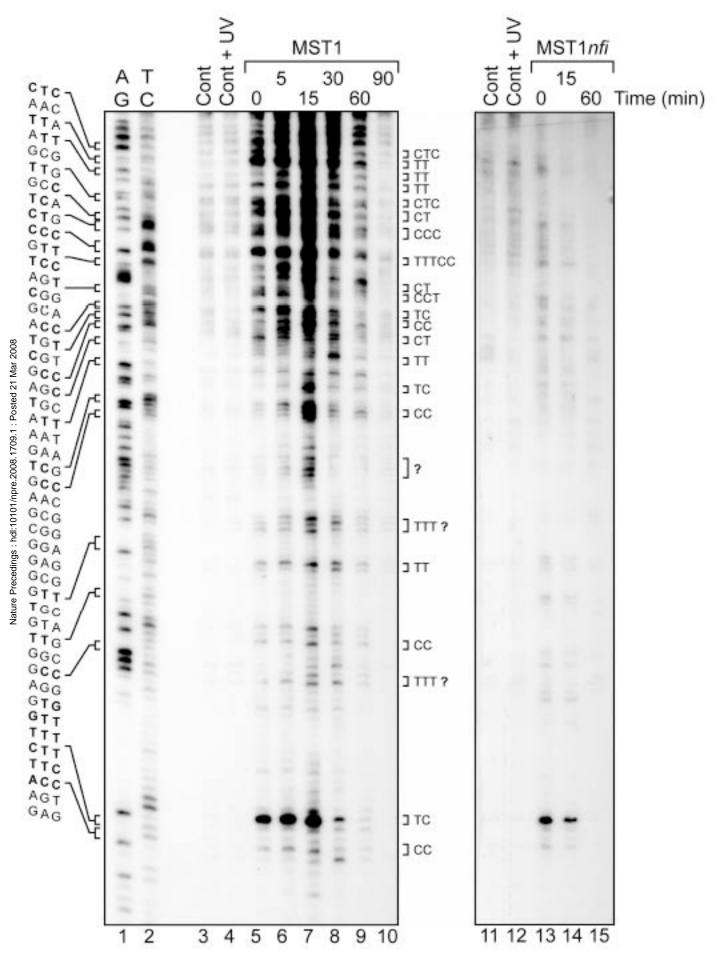
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PHR → SSB

PHR → T + T

a) *uvrA*

b) *uvrB*

c) *uvrC*

d) *nfi*

MST13

MST8

Time (min)

a)

21-mer Primer   5'-32P - - - - - - - GCGT   1   2

65-mer Template   3'-OH - - - - - - CGCAATGTGGT<>TAACAGTG - - - - - - GAC-5'

21   27   29   65

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b)

5   10   20   50   100   dNTP (µM)

Block T2
Block T1
Block

← Primer

1   2   3   4   5

c)

A   G   C   T

29A
28A

1   2   3   4