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Substrate Overlap and Functional Competition between Human Nucleotide Excision Repair and *Escherichia coli* Photolyase and (A)BC Excision Nuclease†

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ABSTRACT: Human cell free extract prepared by the method of Manley et al. (1980) carries out repair synthesis on UV-irradiated DNA. Removal of pyrimidine dimers by photoreactivation with DNA photolyase reduces repair synthesis by about 50%. With excess enzyme in the reaction mixture photolyase reduced the repair signal by the same amount even in the absence of photoreactivating light, presumably by binding to pyrimidine dimers and interfering with the binding of human damage recognition protein. Similarly, the UvrB subunit of *Escherichia coli* (A)BC excinuclease when loaded onto UV-irradiated or psoralen-adducted DNA inhibited repair synthesis by cell-free extract by 75–80%. The opposite was true also as HeLa cell free extract specifically inhibited the photorepair of a thymine dimer by DNA photolyase and its removal by (A)BC excinuclease. Cell-free extracts from xeroderma pigmentosum (XP) complementation groups A and C were equally effective in blocking the *E. coli* repair proteins, while extracts from complementation groups D and E were ineffective in blocking the *E. coli* enzyme. These results suggest that XP-D and XP-E cells are defective in the damage recognition subunit(s) of human excision nuclease.

Wood et al. (1988, 1989) and Sibghat-Ullah et al. (1989) have recently shown that cell-free extract of human cells prepared by the method of Manley et al. (1980) carries out repair synthesis on DNA damaged by UV light, psoralen, and cisplatinum. The repair synthesis activity was absent in all XP cell lines tested, and therefore it was concluded that the DNA adducts were removed by nucleotide excision repair. Efforts in our laboratory to characterize this activity further by fractionation of extracts or by using defined substrates were of limited success. Therefore, we have resorted to alternative methods to define the substrate specificity of the human repair enzyme and its interaction with DNA substrate. In this study we have used *Escherichia coli* and *Saccharomyces cerevisiae* photolyase and *E. coli* (A)BC excinuclease, whose interaction with DNA is well understood (Husain et al., 1987; Baer & Sancar, 1989; Van Houten et al., 1987), to probe the interaction of the human nucleotide excision repair enzyme(s) with DNA. Our findings suggest that about 50% of the repair synthesis by HeLa cell free extract on UV-irradiated DNA is blocked by excess photolyase in the reaction mixture and therefore must be due to the repair of pyrimidine dimers. Similarly, 60–80% of repair synthesis on UV-irradiated or psoralen-adducted DNA can be blocked by UvrB[1] loaded onto

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[1] Abbreviations: UV-DNA, UV-irradiated DNA; UvrA, UvrB, and UvrC, gene products of *uvrA*, *uvrB*, and *uvrC*; CFE, cell-free extract; HMT, 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen; UDS, unscheduled DNA synthesis.

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damaged DNA by UvrA, indicating (i) substantial overlap between substrate specificities of human and *E. coli* excision nucleases, (ii) a lack of cross-complementation between these two enzyme systems, and (iii) cross-species interference of subunits of repair enzymes.

Materials and Methods

Enzymes. HeLa S3 cells were from the Lineberger Cancer Research Center, University of North Carolina. Xeroderma pigmentosum cell lines GM2250 (XP-A) and GM2498 (XP-C) were purchased from Human Genetic Mutant Cell Repository (Camden, NJ). Primary skin fibroblasts, GM38 passage 11 (normal), CRL1157 passage 6 (XP-D), and CRL 1259 passage 12 (XP-E) were the kind gift of M. C. Paterson, Cross Cancer Institute, Edmonton, Canada. The cell-free extracts from these cell lines were prepared by the method of Manley et al. (1980) and were stored at −80 °C in small aliquots until use. The subunits of (A)BC excinuclease were purified as described by Thomas et al. (1985). The *E. coli* DNA photolyase was purified by the method of Sancar and Sancar (1984). The yeast (*S. cerevisiae*) DNA photolyase was a gift from Dr. G. B. Sancar, University of North Carolina, and was purified as described (Sancar et al., 1987).

Substrates. pBR322 plasmid containing UV photoproducts or psoralen adducts was prepared as described previously (Sibghat-Ullah et al., 1989). Briefly, UV-pBR322 was obtained by irradiating DNA at 20 μg/mL with 75 J m^{-2} of 254-nm light, which introduces 5–6 photoproducts. The average number of photoproducts per plasmid was estimated by the transformation assay. Psoralen-adducted DNA was obtained by mixing pBR322 at 50 μg/mL with [^{3}H]HMT (10 Ci/mol, HRI Associates, Emeryville, CA) at 4 μCi/mL and irradiating with 50 kJ m^{-2} of 366-nm light; this treatment introduces 5 psoralen adducts per plasmid. The number of psoralen adducts was calculated from the amount of radioactivity of [^{3}H]HMT, which becomes covalently linked to DNA upon irradiation with near UV light. The 48-mer duplex, which contains a thymine dimer in the middle, was prepared as described previously (Husain et al., 1987, 1988) with either terminal label or internal label at the seventh phosphodiester bond 5′ to the thymine dimer.

Assays. The repair synthesis assay was conducted as follows (Wood et al., 1988; Sibghat-Ullah et al., 1989). The reaction mixture (50 μL) contained 40 mM Hepes, pH 7.9, 50 mM KCl, 8 mM $MgCl_2$, 1 mM dithiothreitol, 0.4 mM EDTA, 200 μg/mL bovine serum albumin, 4% glycerol, 2 mM ATP, 50 μg of phosphoenolpyruvate, 5 units of pyruvate kinase (Sigma), 20 μM each dATP, dGTP, and dTTP, and 8 μM dCTP in addition to 2 μCi of [α-^{32}P]dCTP (6000 Ci/mmol) and 200 ng of pBR322. The reaction was initiated by addition of Manley extract (10–50 μg of protein), and the mixture was incubated at 30 °C for 60–180 min as indicated. After the incubation, the DNA was extracted with phenol and ether, precipitated with ethanol, digested with *Eco*RI restriction endonuclease, and separated on a 1% agarose gel. The gel was dried and autoradiographed. The DNA bands were then cut out, and the radioactivity incorporated into DNA was quantified by scintillation counting.

(A)BC excinuclease was assayed by using the 48-mer substrate that was internally labeled at the seventh phosphodiester bond 5′ to the thymine dimer. The reaction was conducted in 50 μL of repair synthesis buffer which also contained CFE (0–4 μg) when indicated without the dNTPs. Incubation was at 30 °C for 90 min. The DNA was then extracted with phenol, precipitated with ethanol, vacuum-dried, and analyzed on a 12% sequencing gel. The 13-mer excised by the enzyme was cut out of the gel and quantitated by Cerenkov counting.

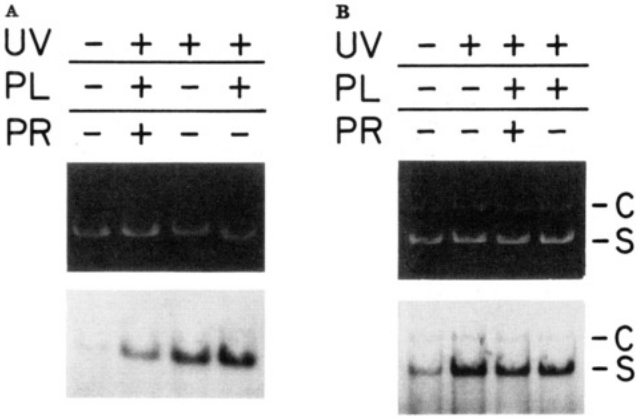

FIGURE 1: Effect of *E. coli* photolyase on nucleotide excision repair in *E. coli* and HeLa cell free systems. (A) *E. coli* repair system. Plasmid pBR322 DNA (200 ng) containing 5–6 UV photoproducts per molecule was incubated in 50 μL of repair synthesis buffer with 400 nM photolyase for 20 min at 23 °C either with (+PR) or without (−PR) photoreactivating light. Then, UVrA (8 nM), UvrB (60 nM), UvrC (30 nM), DNA polymerase I (0.1 unit), and T4 DNA ligase (1 unit) were added, and incubation was continued for another 30 min at 30 °C in the dark. (B) HeLa cell free system. Plasmid pBR322 (200 ng) containing 5–6 UV photoproducts was incubated with photolyase as in the *E. coli* system, and then 25 μg of HeLa CFE was added; repair synthesis was carried out for 3 h in the dark. In both (A) and (B) the DNA was linearized with *Eco*RI endonuclease, separated on 1% agarose gels which were then photographed (top) and autoradiographed (bottom). C, unirradiated M13RF DNA used as internal control; S, substrate pBR322 DNA.

Photolyase also was assayed by using the 48-mer with thymine dimer. The enzyme (20 nM) was incubated with the substrate (~0.1 nM) in repair synthesis buffer for 10 min in the dark and, when indicated, exposed either to two photoreactivating light flashes of 1 ms each from a Vivitar 2500 flash unit or to 366-nm light from two Black Lights (UV Photoproducts) for 20 min at about 20-cm distance at 23 °C.

Results

Inhibition of Repair Synthesis by Photolyase. E. coli photolyase stimulates incision of UV-irradiated DNA by the *E. coli* excision repair enzyme, (A)BC excinuclease (Sancar et al., 1984). This stimulation can be seen in a repair synthesis assay as well (Figure 1 and Table I). Figure 1A shows that in a repair synthesis assay with *E. coli* (A)BC excinuclease and DNA polymerase I photoreactivation of UV-DNA reduces the repair signal by about 50%, while incubation with photolyase in the dark stimulates repair synthesis about 20%; this is in qualitative agreement with the results obtained with the incision assay.

When a similar experiment was performed with *E. coli* photolyase and HeLa cell free extract, the results shown in Figure 1B were obtained. Photolyase reduced the level of repair synthesis by CFE by about 50% regardless of whether the mixture was exposed to photoreactivating light. The simplest interpretation of this result is that pyrimidine dimers are responsible for 50% of repair synthesis on UV-irradiated DNA. When the dimers are eliminated by photoreactivation, the repair synthesis is reduced by half. In the absence of photoreactivating light photolyase binds to pyrimidine dimers and interferes with binding of the human damage recognition protein and thus prevents incision and the subsequent repair synthesis.

Effect of Yeast Photolyase. The results shown in Figure 1 indicate that the human subunit responsible for damage recognition, and thus initiation of repair synthesis, makes

Table I: Effect of *E. coli* and *S. cerevisiae* Photolyases on *E. coli* and Human Nucleotide Excision Repair

photolyase	light	repair synthesis (cpm) *E. coli*	HeLa
no	no	1420	430
E. coli	no	1720	150
E. coli	yes	750	220
no	no	1600	770
yeast	no	500	310
yeast	yes	620	370

[a] The repair synthesis buffer contained the following in 50 μL of repair synthesis buffer. *E. coli* system: 100 ng of UV-pBR322, 1 μg of DNA photolyase, 8 nM UvrA, 60 nM UvrB, 30 nM UvrC, 0.1 unit of DNA polymerase I, and 1 unit of T4 DNA ligase. Repair synthesis was for 1 h at 37 °C. HeLa repair system: 200 ng of UV-pBR322, 1 μg of DNA photolyase, and 24 μg of CFE. Incubation was for 3 h at 30 °C. The background incorporation into undamaged DNA ranged from 50 to 200 cpm and has been subtracted from the values given in the table. The nearly 2-fold variation in the signal of UV-irradiated DNA with HeLa cell free extract is typical of what we see with different CFE preparations. The counts have been rounded off to the nearest ten.

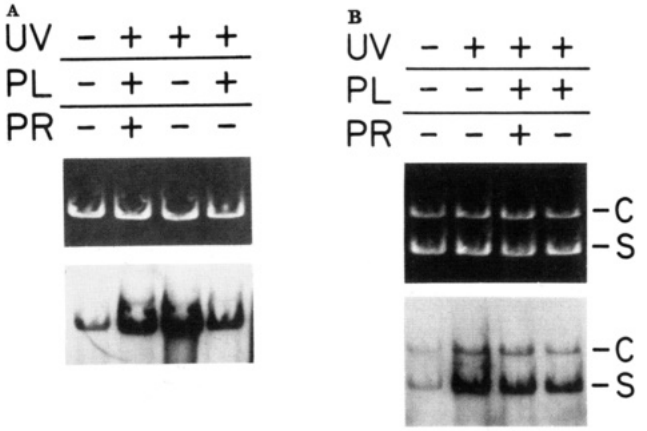

FIGURE 2: Effect of yeast photolyase on excision repair in (A) *E. coli* and (B) HeLa cell free systems. The reaction conditions and method of analysis were as described in Figure 1 except yeast photolyase was substituted for the *E. coli* enzyme.

different contacts with DNA from those of the corresponding subunit (UvrA) of *E. coli* excision nuclease. Sancar and Smith (1989) have recently reported that yeast photolyase inhibits *E. coli* excision repair in vivo and in vitro while stimulating yeast excision repair in vivo. We reasoned that this eukaryotic enzyme may have a similar effect on excision repair of another eukaryote, and therefore we conducted repair synthesis in the presence of yeast photolyase.

The effect of yeast photolyase on repair synthesis is shown in Figure 2, and the results are summarized in Table I. The inhibition of repair synthesis of the *E. coli* system is in agreement with Sancar and Smith (1989). With the human system, photoreactivation with yeast photolyase reduced the signal by about 50% as expected. Under nonphotoreactivation conditions as well yeast photolyase inhibited repair synthesis in a manner similar to the *E. coli* photolyase. Thus, it appears that the stimulatory effect of yeast photolyase either is species specific or may apply to closely related species but it does not extend to human excision repair. The inhibition of repair synthesis by *E. coli* and yeast photolyases is specific for UV-irradiated DNA. Neither enzyme has any effect on repair synthesis on psoralen-damaged DNA at the enzyme concentrations used in these experiments (Table II). We thus conclude that both photolyases inhibit repair synthesis by binding to pyrimidine dimers and blocking access to the substrate by the damage recognition subunit of human excision nuclease. Note that both photolyase and (A)BC excinuclease inhibition experiments were conducted with substrates with subsaturating amounts of adducts (Sibghat-Ullah et al., 1989) such that any decrease in the number of available damage sites results in a proportional decrease in the signal.

Table II: Effect of *E. coli* and Yeast Photolyases (in the Dark) on Repair Synthesis by HeLa CFE on DNA Damaged by UV or Psoralen

	repair synthesis cpm	% inhibition
expt 1		
UV-DNA	240	
UV-DNA + EPL	70	70
HMT-DNA	260	
HMT-DNA + EPL	270	0
expt 2		
UV-DNA	210	
HMT-DNA	100	
HMT-DNA + YPL	115	0

[a] The reaction mixtures contained 200 ng of pBR322 with either ~10 UV photoproducts or 6 HMT adducts, 1 μg of photolyase (where indicated), and 24 μg of CFE; incubation was for 3 h at 30 °C. The background synthesis, 110 cm on "nondamaged" DNA, has been subtracted from the values given in the table. All the reactions were conducted under yellow light ("in the dark"). EPL and YPL, *E. coli* and yeast photolyase, respectively.

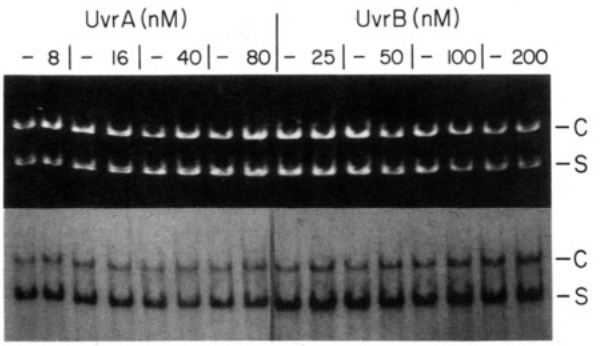

FIGURE 3: Lack of effect of UvrA and UvrB separately on repair synthesis by HeLa CFE. Repair synthesis was conducted on 200 μg of UV-irradiated DNA in the presence of the indicated amounts of either UvrA or UvrB proteins and 24 μg of HeLa CFE. Incubation was for 2 h at 30 °C.

Inhibition of Repair Synthesis by (A)BC Excinuclease. The subunits of *E. coli* (A)BC excinuclease function in an orderly and coordinate manner to remove DNA adducts. UvrA is the damage recognition subunit; it binds to DNA as an A_2 dimer, delivers the UvrB subunit to the damage site, and dissociates from the site. The UvrB–DNA complex is extremely stable ($t_{1/2}$ = 100 min), and upon binding to this complex the UvrC subunit leads to the dual incisions characteristic of the enzyme (Orren & Sancar, 1989).

We wished to know if the UvrA and/or UvrB subunits could substitute for their functional counterparts from human cells or if they would interfere with them. We therefore conducted repair synthesis in the presence of UvrA, UvrB, and UvrA plus UvrB proteins. Figure 3 shows that UvrA up to 80 nM and UvrB up to 200 nM do not have any effect on repair synthesis by CFE. The fact that UvrA has no effect on repair synthesis indicates that either (i) the UvrA analogue is not limiting or (ii) UvrA does not substutite for the analogous protein in this system. Considering the second possibility to be the case, one may expect to see inhibition of repair by UvrA. We suspect that this is not observed because the UvrA–DNA complexes are very transient ($t_{1/2}$ = 5 s), and therefore even in the presence of a high concentration of UvrA protein, the human

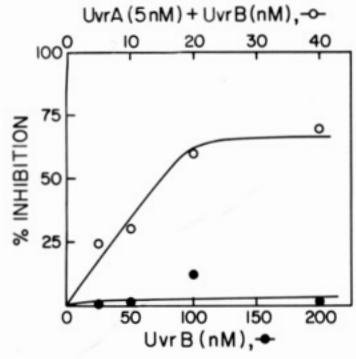

FIGURE 4: Inhibition of human nucleotide excision by UvrB loaded onto UV-DNA. One microgram of UV-pBR322 was incubated at 37 °C for 15 min with 25 nM UvrA and 25–200 nM UvrB in 50 μL of reaction mixture containing 50 mM Tris·HCl, pH 7.5, 60 mM KCl, 2 mM ATP, and 1 mM dithiothreitol. Then 10 μL of this mixture was added to 50 μL of repair synthesis buffer along with 24 μg of CFE, and repair synthesis was carried out for 2 h at 30 °C. The concentrations of UvrA and UvrB indicated in the figure are the final concentrations in the repair synthesis reaction. The percent inhibition was calculated relative to the repair synthesis carried out in the absence of Uvr proteins. The data points for UvrB alone (●) were taken from Figure 3.

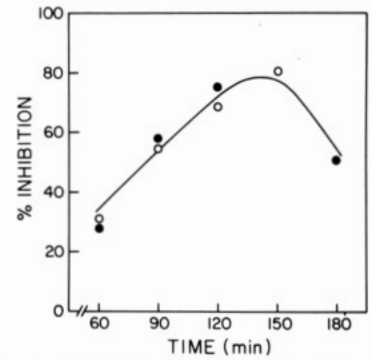

FIGURE 5: Kinetics of inhibition of repair synthesis by preloaded UvrB. One microgram of pBR322 containing either 5–6 UV photoproducts (○) or 5 psoralen monoadducts (●) was incubated at 37 °C for 15 min with 25 nM UvrA and 200 nM UvrB in 50 μL of 50 mM Tris·HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, and 1 mM dithiothreitol. The repair synthesis was initiated by adding 10 μL of this mixture to 50 μL of repair synthesis buffer containing 24 μg of CFE and was stopped at the indicated time intervals, and the level of inhibition compared to reactions conducted in the absence of UvrB was calculated. At less than 60 min of repair synthesis the signal was too weak to allow a quantitative analysis.

damage recognition protein can compete effectively by binding to the damage more tightly than does UvrA. The lack of effect of UvrB on repair synthesis is to be expected as this protein does not interact with DNA measurably in the absence of UvrA.

When repair synthesis was conducted in the presence of catalytic amounts of UvrA and increasing concentrations of UvrB, the results shown in Figure 4 were obtained. With increasing amounts of UvrB an increasing fraction of repair synthesis was inhibited. The inhibition reached a maximum of about 60% at UvrB concentrations known to be saturating all damage sites (Orren & Sancar, 1989). In other experiments with UV-irradiated DNA up to 80% of repair synthesis was inhibited. Thus, it appears that UvrB is loaded onto pyrimidine dimers as well as other photoproducts (presumably 6–4 photoproducts) and that the presence of UvrB on the photoadducts interferes with binding of the human repair protein. The inhibition is never 100% because the UvrB–DNA complexes dissociate with a $k_{off} = 1.1 \times 10^{-4}$ s^{-1}, and thus substrate becomes available for the human repair enzyme during the long incubation period required to achieve repair synthesis.

We reasoned that if the residual repair synthesis occurring when all sites were occupied by UvrB was due to dissociation of UvrB during the assay period, then the inhibition of repair synthesis would be less over longer time periods as UvrB–DNA complexes decayed with $t_{1/2} = 100$ min. Saturating amounts of UvrB were loaded onto UV-irradiated or psoralen-adducted DNA with catalytic amounts of UvrA, and then the complexes were diluted into repair synthesis buffer to lower the UvrA concentration so that no significant reloading would occur. CFE was then added to the reaction mixture, and repair synthesis was measured as a function of time. The result is shown in Figure 5. Maximum inhibition was obtained with both UV-irradiated and psoralen-damaged DNA when repair synthesis was carried out for 120–150 min. This result was somewhat unexpected because a higher fraction of adducts is bound by UvrB at earlier time points. It is clear, however, that at 120–150 min repair synthesis is inhibited by 75–80%. Afterward, the inhibition is less, presumably because UvrB dissociates from the damaged DNA.

Inhibition of Photolyase by CFE. If photolyase and (A)BC excinuclease inhibit repair synthesis by binding to pyrimidine

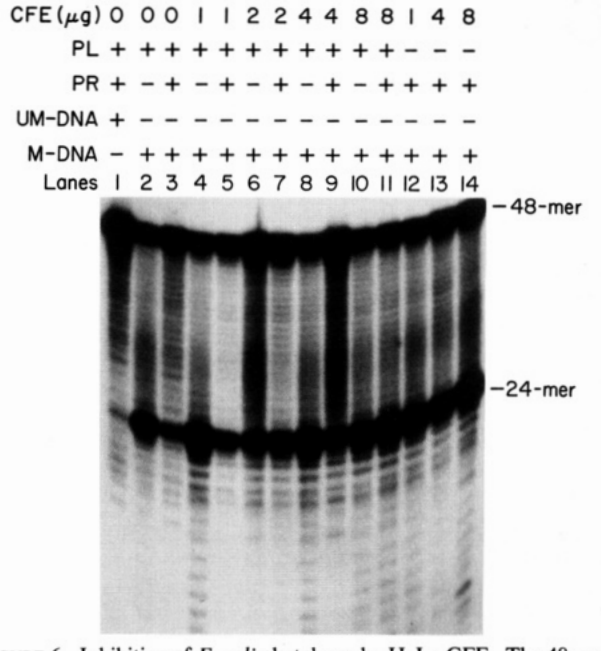

FIGURE 6: Inhibition of *E. coli* photolyase by HeLa CFE. The 48-mer substrate (3 ng) with label at the 5′ end of the dimer-containing strand was incubated in 50 μL of repair synthesis buffer at 30 °C for 30 min with the indicated amounts of CFE. Photolyase was added to 37 nM and incubation continued for another 30 min in the dark. Then the samples were exposed to two intense light flashes from Vivitar 2500 flash units. The DNA was extracted with phenol and hen digested with ca. 10 units of T4 endonuclease V, and the reaction products were analyzed on a 12% sequencing gel. Note that neither the photolyase nor the T4 endonuclease V reactions were complete, accounting for the presence of the 24-mer and the 48-mer in the control reactions.

dimers and blocking access by the human damage recognition protein, then it should be possible to inhibit these enzymes by first incubating the substrate with CFE. Figure 6 shows that such is the case for *E. coli* photolyase. A 48-mer duplex with a single thymine dimer (Husain et al., 1988) was preincubated with increasing concentrations of CFE (note, no specific excision by CFE is observed on this substrate due to low enzyme activity), excess photolyase was added, and then the mixture was either kept in the dark or exposed to two flashes of photoreactivating light. Finally, the DNA was treated with T4 endouclease V and analyzed on a sequencing gel (Figure 6).

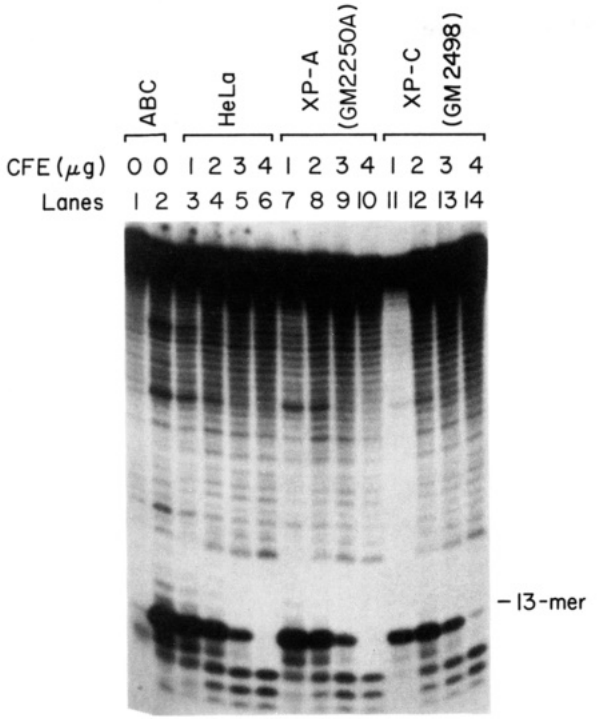

FIGURE 7: Inhibition of (A)BC excinuclease with extracts from normal and XP cells. The 48-mer substrate was incubated with the indicated amounts of CFE from HeLa, XP-A, or XP-C cells for 30 min, and then UvrA (8 nM), UvrB (120 nM), and UvrC (30 nM) were added; incubation was continued for another 2 h at 30 °C.

With excess photolyase, the photorepaired DNA becomes resistant to T4 endonuclease V, and the intensity of the T4 endo V generated 24-mer seen on the gel diminishes (lanes 3 and 5). However, if photoreactivation is carried out in the presence of CFE, photolyase is inhibited and as a consequence the intensity of the T4 endonuclease incision band increases (Figure 6, compare lane 3 to lanes 7, 9, and 11).

Inhibition of (A)BC Excinuclease by CFE. Using the 48-mer substrate, we also investigated the effect of CFE on (A)BC excinuclease. (A)BC excinuclease incises on both sides of the dimer, and a 13-mer is excised (Sancar & Rupp, 1983). We wished to know if CFE would inhibit (A)BC excinuclease by interacting specifically with the substrate. We conducted the (A)BC excinuclease reaction in the presence of increasing amounts of CFE from wild-type (HeLa) and XP-A and XP-C group human cells. The results are shown in Figure 7. Extracts from all three cell lines inhibited the excision reaction in a concentration-dependent manner, the inhibition being near total at 80 μg/mL cell extracts (lanes 6, 10, and 14). A plausible explanation for these results is that the damage recognition subunits bind to the substrate and thus make it inaccessible to (A)BC excinuclease. If that is the case, then it follows that XP-A and XP-C cells are not defective in the damage recognition protein. It was also possible, however, that the inhibition we observed was caused by the nonspecific binding of numerous DNA binding proteins present in the CFE. To address that possibility, we investigated the effect of CFE on digestion of the unmodified 48-mer by *Eco*RI and *Bgl*II restriction endonucleases. We did not detect any inhibition up to 160 μg/mL of CFE (data not shown). While the experiments with restriction enzymes provide some support for our claim of specific inhibition, the simplicity of these restriction enzymes compared to the complexity of (A)BC excinuclease makes a definite conclusion impossible. Therefore, we decided to conduct "order of addition" experiments in an attempt to gather further evidence for specific inhibition.

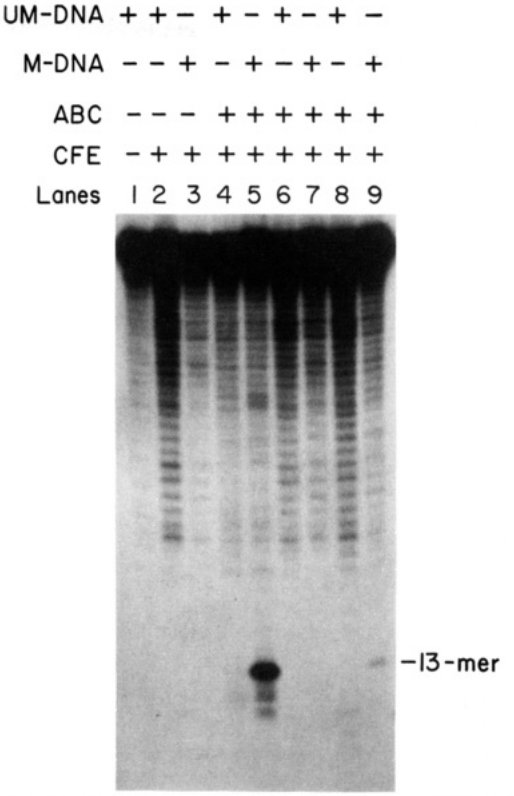

FIGURE 8: Effect of order of addition on inhibition of (A)BC excinuclease by CFE. The 48-mer duplex without (UM-DNA) or with thymine dimer (M-DNA) was subjected to the following treatments before analysis on a 12% sequencing gel. (Lane 1) UM-DNA incubated for 2 h in repair synthesis buffer; (lane 2) UM-DNA incubated with 8 μg of CFE for 2 h; (lane 3) same as lane 2 except M-DNA was used; (lane 4) UM-DNA incubated with 8 nM UvrA, 120 nM UvrB, and 30 nM UvrC for 30 min and then with 8 μg of CFE for 2 h; (lane 5) same as lane 4 except M-DNA was used; (lane 6) UM-DNA incubated for 30 min with 8 μg of CFE, and then UvrA, UvrB, and UvrC were added and incubation was continued for another 2 h; (lane 7) same as lane 6 except M-DNA was used; (lane 8) UM-DNA incubated with 8 nM UvrA and 120 nM UvrB for 30 min and then 8 μg of CFE was added followed immediately by addition of UvrC to 30 nM and incubation for another 2 h at 30 °C; (lane 9) same as lane 8 except M-DNA was used.

Order of Addition Experiments. (A)BC excinuclease is a complex ATP-dependent enzyme (Sancar & Sancar, 1988). The nuclease assembles on DNA in successive steps; the ATPase and DNA binding subunit UvrA delivers UvrB to the damage site and then dissociates; UvrC binds to the stable UvrB–DNA complex, and then the two incisions are made (Orren & Sancar, 1989). We wished to identify the particular step that was inhibited by the human protein(s). When the extract was first preincubated with substrate and UvrA, UvrB, and UvrC were added, no excision by (A)BC excinuclease was observed (Figure 8, lane 6); however, if the substrate was first incubated with UvrA plus UvrB followed by the addition of CFE before UvrC was added, specific, albeit diminished, excision was observed (Figure 8, lane 9). Furthermore, the UvrB–DNA complexes formed were quite stable in the presence of CFE. Addition of UvrC either immediately after CFE or 1 h after incubation of UvrB–DNA complex with CFE resulted in about the same level of excision (Figure 9), in agreement with the finding that the UvrB–DNA complex is long-lived with a $t_{1/2}$ = 100 min (Orren & Sancar, 1989). We interpret these results to mean that CFE contains a damage recognition protein which binds to the substrate and interferes with binding of UvrA and loading of UvrB. However, there is substantial inhibition of excision even when the extract is

Table III: Inhibition of (A)BC Exinuclease by Human CFE

	relative excision by (A)BC exinuclease[a]											
	HeLa		GM38		XP-A		XP-C		XP-D		XP-E	
CFE (μg)	expt 1	expt 2	expt 1	expt 2	expt 1	expt 2	expt 1	expt 2	expt 1	expt 2	expt 1	expt 2
0	100	100	100	100	100	100	100	100	100	100	100	100
1	75	72	100	90		70		60	88	89	60	73
2	43	28	50	56		53	32	40	73	84	58	62
3	10	0	6	5	0	13	9	5	35	52	18	32
4	0	0	5	5	0	0	0	0	30	36	10	22

[a] These values were obtained from the gels shown in Figures 7 and 10 and from additional experiments conducted under similar conditions.

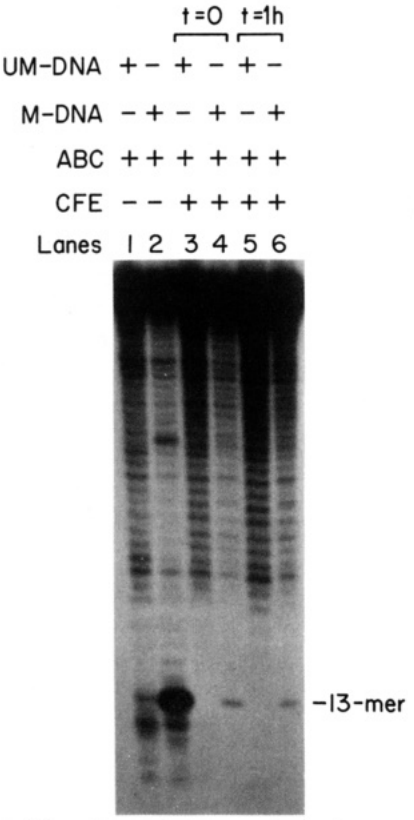

FIGURE 9: Stability of UvrB–DNA complex in the presence of CFE. The 48-mer (3 ng) was incubated with 8 nM UvrA and 120 nM UvrB for 30 min at 30 °C, and then 8 μg of CFE was added to the mixture either following immediately (t = 0) by UvrC and incubation for 1 h or, after 1 h of incubation with CFE (t = 1 h), addition of UvrC and incubation for an additional 1 h. The samples were then analyzed on 12% sequencing gel.

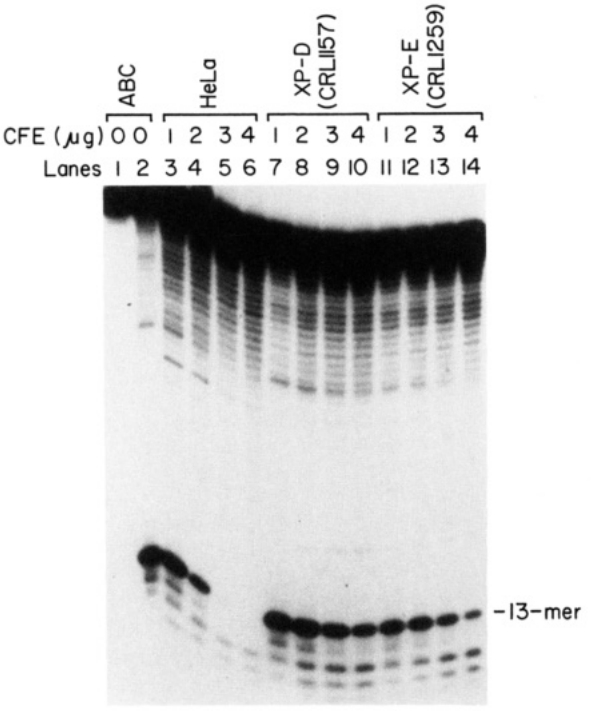

FIGURE 10: Effect of CFE from XP-D and XP-E cells on *E. coli* (A)BC excinuclease. The 48-mer substrate was incubated with the indicated amounts of CFE followed by UvrA, UvrB, and UvrC under the same conditions as in Figure 7, and the reaction products were analyzed on a 10% sequencing gel.

added after UvrB is loaded onto the substrate (Figure 9, compare lane 2 to lanes 4 and 6). We think that this inhibition is caused by nonspecific binding of the many DNA binding proteins present in such an extract. Nevertheless, the differential effect of excision on preloaded DNA suggests that a fraction of the inhibition is specific.

Lack of Specific Inhibition by CFE from XP-D and XP-E Cell Lines. It has recently been reported that XP-E cells are defective in the damage recognition subunit of human excision nuclease (Chu & Chang, 1988; Patterson & Chu, 1989) as evidenced by the lack of a retarded band in band-shift assays with UV-irradiated DNA. If the interference of CFE with ABC excinuclease is indeed due to binding of XP-E protein, then extracts from these cells should behave differently from those of normal cells or cells from other complementation groups. Therefore, in addition to XP-A and XP-C shown in Figure 7, we tested XP-D and XP-E to show the unique behavior of the XP-E group. Unexpectedly, we found that CFE from both complementation groups failed to inhibit (A)BC excinuclease specifically (Figure 10). However, the cells used for these experiments were primary fibroblasts as opposed to the XP-A and XP-C cell lines, which are lymphoid cells immortalized with Epstein–Barr virus. To eliminate the possibility of an artifactural difference due to different tissue and/or type of cell, we used a primary fibroblast (GM38) of a normal individual as a control. The level of inhibition observed was comparable to that of HeLa cells or lymphoid cells of XP-A and XP-C origin. The results of inhibition experiments conducted with different cell lines are summarized in Table III. As is apparent quantitatively, the level of inhibition varies from experiment to experiment, but qualitatively the same trend is observed under a variety of different experimental conditions. Therefore, we feel that all these data taken together are suggestive that part of the inhibition of (A)BC excinuclease by CFE is due to specific binding of damage recognition proteins which are missing in XP-D and XP-E cell lines.

Discussion

We have found that *E. coli* and *S. cerevisiae* photolyases and *E. coli* (A)BC excinuclease interfere with the human nucleotide excision repair system and conversely that the human repair system interferes with *E. coli* photolyase and (A)BC excinuclease. These observations have some fundamental as well as practical implications.

The fact that *E. coli* photolyase reduces the repair synthesis signal by about 50% with or without photoreactivating light

suggests that pyrimidine dimers, which make up 80–90% of UV photoproducts, are responsible for this fraction of repair, while the 6–4 photoproducts, which make up 10–15% of photoproducts, are responsible, along with the other minor photoproducts, for the other half (Sibghat-Ullah et al., 1989; Wood, 1989). Assuming that both pyrimidine dimers and 6–4 photoproducts are recognized by the same protein and that the repair patch synthesized upon their removal is of the same size, it appears that in human cells the 6–4 photoproducts are removed 5 times more efficiently than pyrimidine dimers, as is the case in *E. coli* (Myles et al., 1987, and data not shown). The cause of this preferential repair is unknown at present. Model building and NMR studies suggest that 6–4 photoproducts distort DNA more severely compared to pyrimidine dimers (Rycyna & Alderfer, 1986). However, at least in *E. coli*, no correlation has been found between the severity of the distortion induced by a lesion and that lesion's susceptibility to repair by nucleotide excision (Lin & Sancar, 1989). It may also be argued that some of the repair synthesis on UV-irradiated DNA may be the result of removal of thymine and cytosine glycols by glycosylases. While base excision repair may be partly responsible for the repair synthesis signal, it must be pointed out that thymine glycols are excellent substrates for *E. coli* excision nuclease, the (A)BC excinuclease (Lin & Sancar, 1989), and therefore a distinction between base excision and nucleotide excision based on substrate structure is impossible.

The inhibition of human nucleotide excision repair by photolyases and loaded UvrB indicates overlap between the binding sites of these proteins and the human damage recognition subunit. While the contact sites on DNA by photolyase are known at atomic resolution (Husain et al., 1989; Baer & Sancar, 1989), less is known about the interaction of (A)BC excinuclease with substrate. It is only known that UvrB makes a 19-bp DNase I footprint around the adduct (Van Houten et al., 1988). Furthermore, for enzyme interference in binding it is not necessary that the two proteins make the same contacts. Yeast photolyase, which makes essentially the same phosphodiester bond backbone as well as major and minor groove contacts as the *E. coli* photolyase (Baer & Sancar, 1989; Sancar, 1990), inhibits (A)BC excinuclease in contrast with the *E. coli* enzyme which stimulates the excision nuclease. The most likely explanation is that the two proteins are sufficiently different in shape such that the yeast enzyme interferes with binding of the damage recognition subunit, UvrA, by steric hindrance. With these considerations in mind then, the significance of inhibition of the human repair by the *E. coli* and yeast enzymes might be considered to be of marginal importance. However, the level of substrate overlap between the various repair enzymes allows us to make certain conclusions regarding the substrate specificity and substrate range of the human excision nuclease(s). Thus, our study has provided biochemical evidence to support the genetic data that *E. coli* and human nucleotide excision nucleases have similar substrate range.

The inhibition of *E. coli* photolyase and (A)BC excinuclease is not more revealing regarding the interaction of the human enzyme with substrate than the inhibition of the human nuclease by the *E. coli* enzymes. However, the enzyme interference assay has provided us a means of testing XP cell lines for the presence of damage recognition subunit. We can thus state that XP-A and XP-C mutants have the damage recognition subunit. In contrast, XP-D and XP-E mutants seem to be detective in the protein(s) that bind damage. Our finding with XP-E is in agreement with that of Chu and Chang (1988), who, using gel retardation, found XP-E cells to be defective in a protein that specifically binds UV-irradiated DNA. However, they also reported XP-D cells to be normal in binding activity. It is conceivable that the nature of binding by the XP-D gene product is such that its association with DNA cannot be detected by gel retardation but can be detected by our enzyme interference assay. Indeed, binding of UvrB to DNA cannot be demonstrated by gel retardation, but it is demonstrable by other assays (Orren & Sancar, 1989) including interference with repair enzymes from other species as shown in this study.

From a practical standpoint, our results suggest that caution must be exercised in interspecies gene or protein complementations. Photolyase and (A)BC excinuclease are able to function in the presence of moderate concentrations of cell-free extract; however, both are inhibited at higher concentrations. Although some of the inhibition is nonspecific, a significant fraction is specific, and it is likely to occur in vivo as well. Complementation of the XP defect has been achieved by inserting the T4 *denV* gene (Valerie et al., 1985) or protein into these cells. Similarly, it was found (Zwetsloot et al., 1985, 1986a) that microinjected yeast photolyase reduced the residual unscheduled DNA synthesis in xeroderma pigmentosum groups C, F, and I but had no effect on UDS in XP-A, -D, -E, and -H. The authors speculated that in these latter groups the defective repair enzymes interfered with binding of photolyase. It is indeed conceivable that a mutation in a DNA binding protein would eliminate its normal function without interfering with its DNA binding affinity (Myles and Sancar, unpublished results). Similarly, transfection of XP-A cells with the *uvrA* gene resulted in production of UvrA protein but not complementation of the repair defect (Dickstein et al., 1988). Microinjection of UvrA, UvrB, UvrC, and UvrD (helicase II) proteins also failed to elicit any UDS in XP-A, -C, and -H cells (Zwetsloot et al., 1986b). It appears that in none of these experiments was the possibility of repair inhibition by the microinjected proteins considered. In light of the results reported in this paper, it is conceivable that the microinjected proteins not only failed to complement but actually inhibited repair synthesis. Finally, our experiments show that the in vitro repair synthesis assay can be used to test interspecies complementation (or interference), and because of its simplicity this assay should be considered an alternative to the more laborious and technically difficult transfection and microinjection assays in testing genes and proteins for interspecies complementation.

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Kinetics and Thermodynamics of Oxygen and Carbon Monoxide Binding to the T-State Hemoglobin of *Urechis caupo*†

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ABSTRACT: The tetrameric hemoglobin from *Urechis caupo* is nearly ideal for studying ligation to the T-state. Our previous EXAFS study had shown that the Fe is displaced 0.35 Å from the mean plane of the porphyrin in the HbCO derivative. We have carried out detailed kinetic studies of oxygen and CO ligation as a function of temperature in order to characterize both the kinetics and thermodynamics of ligation in this hemoglobin. The entropy change associated with ligation essentially corresponds to simple immobilization of the ligand and is virtually the same as that we have determined for leghemoglobin, an extreme R-state-type hemoglobin. The low ligand affinities thus derive from small enthalpies of ligation, which can be correlated with the large out of plane displacement of the Fe. Only oxygen pulse measurements revealed kinetic evidence for cooperative oxygen binding, but a direct measurement of oxygen binding gave a Hill number of 1.3. An allosteric analysis gave $L = 2.6$ and $c = 0.048$ (oxygen) and $c = 0.77$ (CO). The higher affinity state in this weakly cooperative hemoglobin is denoted T*, and it is for this state that thermodynamic quantities have been determined. The small differences between T and T* in CO binding were nevertheless sufficient to allow us to measure by flash photolysis the rate of the T* → T conformational change in terms of an allosteric model. The half-time for this transition was calculated to be 8-14 ms at 20 °C.

The hemoglobin of the echiuroid *Urechis caupo* is a tetramer of molecular weight 57 600 for which oxygen binding has been reported to be noncooperative and of low affinity (Garey & Riggs, 1984; Sima, 1979) or weakly cooperative (Mangum et al., 1983) and which we have characterized as a T-state hemoglobin (Schreiber & Parkhurst, 1984) with an unusually large displacement of the Fe out of the mean plane of the heme in the HbCO form (Chance et al., 1986). Garey and Riggs (1984) have recently reported that there is one predominant form of the tetramer consisting of nearly identical subunits. A recent X-ray crystallographic study at 5-Å resolution reported that the tetramer has unusual subunit contacts, giving rise to an "inside-out" quaternary structure with the G/H helices located on the outer surface (Kolatkar et al., 1988). We wished to extend our previous kinetic studies (Sima, 1979) to obtain the thermodynamics of oxygen and CO ligation in order to characterize a naturally occurring T-state hemoglobin. Oxygen and CO ligand binding kinetic experiments were carried out as a function of temperature to allow calculation of $\Delta H°$ and $\Delta S°$. We report here the oxygen and CO association and dissociation rate constants, equilibrium constants, and the activation energies and the standard-state changes in free energy, enthalpy, and entropy of ligation. In the course of these studies, we found from both kinetic and equilibrium experiments that the protein showed weak cooperativity. We were not only able to characterize this behavior in terms of

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