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A New Mechanism for Repairing Oxidative Damage to DNA: (A)BC Excinuclease Removes AP Sites and Thymine Glycols from DNA†

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ABSTRACT: *Escherichia coli* (A)BC excinuclease is the major enzyme responsible for removing bulky adducts, such as pyrimidine dimers and 6–4 photoproducts, from DNA. Mutants deficient in this enzyme are extremely sensitive to UV and UV-mimetic agents, but not to oxidizing agents, or ionizing radiation which damages DNA in part by generating active oxygen species. DNA glycosylases and AP[1] endonucleases play major roles in repairing oxidative DNA damage, and thus it has been assumed that nucleotide excision repair has no role in cellular defense against damage by ionizing radiation and oxidative damage. In this study we show that the *E. coli* nucleotide excision repair enzyme (A)BC excinuclease removes from DNA the two major products of oxidative damage, thymine glycol and the baseless sugar (AP site). We conclude that nucleotide excision repair is an important cellular defense mechanism against oxidizing agents.

Active oxygen species (the superoxide radical, $O_2^{\bullet-}$, hydroxyl radical, $OH^{\bullet}$, and hydrogen peroxide, H_2O_2) generated by incomplete reduction of oxygen during aerobic metabolism are serious threats to cellular integrity. All aerobic organisms appear to have molecular mechanisms to protect themselves against and to repair the damage caused by these agents. The protective enzymes include superoxide dismutase, which converts $O_2^{\bullet-}$ into H_2O_2, and catalase, which converts H_2O_2 into molecular oxygen and H_2O, as well as specific peroxidases [see Fridovich (1989)]. In addition to these protective measures there are several enzymes that are involved in repairing the oxidative damage to DNA. In *Escherichia coli* it has been shown that exonuclease III (Demple et al., 1983), endonucleases III and IV (Cunningham et al., 1986), and RecA protein (Imlay & Linn, 1986) play important roles in repairing DNA damaged by oxidative damage, as well as by ionizing radiation which damages DNA mainly through generation of $OH^{\bullet}$ radicals [see Von Sonntag (1987) for a review]. Enzymes that repair oxidative DNA damage (redoxyendonuclease) have also been found in yeast (Gossett et al., 1988) and in bovine and human cells (Doetsch et al., 1987).

The repair of damage caused by either ionizing radiation or active oxygen species is accomplished by removal of the saturated (e.g., thymine glycol) or fragmented (urea, methyltartronylurea) base by so-called redoxyendonucleases

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[1] Abbreviations: AP, apurinic/apyrimidinic; TG, thymine glycol; AP-DNA, DNA with apurinic/apyrimidinic sites; TG-DNA, DNA containing thymine glycols; Nth, *E. coli* endonuclease III; Nfo, *E. coli* endonuclease IV.

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which include the *E. coli* endonuclease III (Katcher & Wallace, 1983; Breimer & Lindahl, 1984) and its functional homologues in other organisms. Then the remaining sugar (AP site) is removed, leaving a single nucleotide gap in DNA [base excision repair; see Wallace et al. (1988) and Sancar and Sancar (1988) for recent reviews]. In addition, 3′-phosphoglycolaldehyde (PGA) diesterases have been identified in both *E. coli* (Bernelot-Moens & Demple, 1989) and yeast (Johnson & Demple, 1988a,b) which remove 3′-PGA esters produced as a result of base fragmentation and strand scission by oxidative damage or ionizing radiation.

Nucleotide excision repair in *E. coli* entails the removal of a damaged base by incision of the eighth phosphodiester bond 5′ and the fifth phosphodiester bond 3′ to the damage site (Sancar & Rupp, 1983; Sancar & Sancar, 1988). The *uvrA*, *uvrB*, and *uvrC* genes encode the enzyme responsible for making these incisions. The *uvr* genes were identified by their involvement in repairing DNA damage caused by nonionizing radiation, typically UV of 254 nm (Howard-Flanders et al., 1966). Subsequently, it was found that mutations in any of these three genes made cells sensitive to a number of chemicals that make bulky adducts in DNA, but not to ionizing radiation (Howard-Flanders & Boyce, 1966) or to H_2O_2 (Imlay & Linn, 1987). However, we have now found that the mixture of UvrA, -B, and -C proteins [(A)BC excinuclease] removes the two major mutagenic and lethal (Loeb, 1985; Clark et al., 1987) products of ionizing radiation and oxidizing agents, thymine glycol and the AP sites. These results are of interest not only for revealing a new pathway for repairing oxidative damage but also for raising important questions regarding the damage recognition mechanism of (A)BC excinuclease.

Materials and Methods

DNA and Enzymes. Plasmid pBR322 DNA was isolated from *E. coli* AB2437/pBR322 grown in K medium containing [^{3}H]TdR at 5 μCi/mL (Sancar et al., 1985) by two successive centrifugations in ethidium bromide–CsCl gradients. The DNA was 80–90% superhelical and had a specific activity of 1.1×10^4 cpm/μg. For linear substrate we used the (*Bam*HI–*Eco*RI)$_{375}$ fragment of pBR322 that was terminally labeled with [γ-^{32}P]ATP and polynucleotide kinase (New England Biolabs, Beverly, MA) or with [α-^{32}P]dATP and Klenow fragment (Boehringer-Mannheim) and then digested with *Hae*III (New England Biolabs) to yield the (*Eco*RI–*Hae*III)$_{174}$ fragment labeled at the *Eco*RI terminus.

The three subunits of (A)BC excinuclease, UvrA, UvrB, and UvrC, were purified as described elsewhere (Sancar & Rupp, 1983). *E. coli* endonuclease IV (Levin et al., 1988) was kindly provided by Dr. B. Demple (Harvard University), and *E. coli* endonuclease III was a gift of Dr. R. P. Cunningham (SUNY, Albany).

Preparation of Substrates. To prepare the thymine glycol containing substrate, plasmid DNA in 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, and 0.04% OsO_4 was heated at 65 °C for 30 min; the terminally labeled fragment was heated at 70 °C for 90 min in 0.07% OsO_4 (Kow & Wallace, 1987). The apurinic substrate was prepared by heating DNA in 10 mM sodium citrate (pH 5.2) and 100 mM NaCl for 40 min (plasmid) or 25 min (terminally labeled DNA). Following modification, DNA was precipitated with ethanol and resuspended in 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, and 1 mM EDTA (Breimer & Lindahl, 1984). The average number of AP sites or thymine glycols in DNA was determined by limit digestion with Nfo or Nth, respectively.

Repair Enzyme Assays. We used two assays to investigate the effect of (A)BC excinuclease on DNA containing thymine glycols or AP sites. The plasmid nicking assay was for determining whether or not these modifications constituted substrates for the enzyme, and the assay on terminally labeled fragment was for determining the site of incision relative to the modification site. The plasmid incision assay reaction mixture contained (unless specified otherwise), in 200 μL, 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM ATP, 0.6 pmol of pBR322, 50 μg/mL bovine serum albumin, and when indicated 8 nM UvrA and 180 nM UvrB. The mixture was incubated at 37 °C for 15 min, and then either UvrC was added to 20 nM or endonuclease III or endonuclease IV to 0.2 or 0.4 nM, respectively. The mixture was incubated at 37 °C; at time intervals 12.5-μL aliquots were taken and mixed with loading dye to achieve final concentrations of 20 mM EDTA and 0.05% sodium dodecyl sulfate. The reaction products were separated on 1% agarose gels, and from the fraction of DNA remaining superhelical the average number of nicks per plasmid was calculated by using Poisson distribution of nicks (Orren & Sancar, 1989).

To determine the incision sites of endonuclease III, endonuclease IV, or (A)BC excinuclease, approximately 3000 cpm of terminally labeled DNA was incubated for 30 min at 37 °C in 25 μL of (A)BC excinuclease buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM ATP, and 50 μg/mL bovine serum albumin) with 0.2 nM endonuclease III, 0.4 nM endonuclease IV, or 8 nM UvrA, 180 nM UvrB, and 20 nM UvrC. The samples were then dried, resuspended in 25 μL of formamide dye, and heated for 60 s at 90 °C before being loaded onto a 8% sequencing gel. Both AP-DNA and TG-DNA were also hydrolyzed by heating

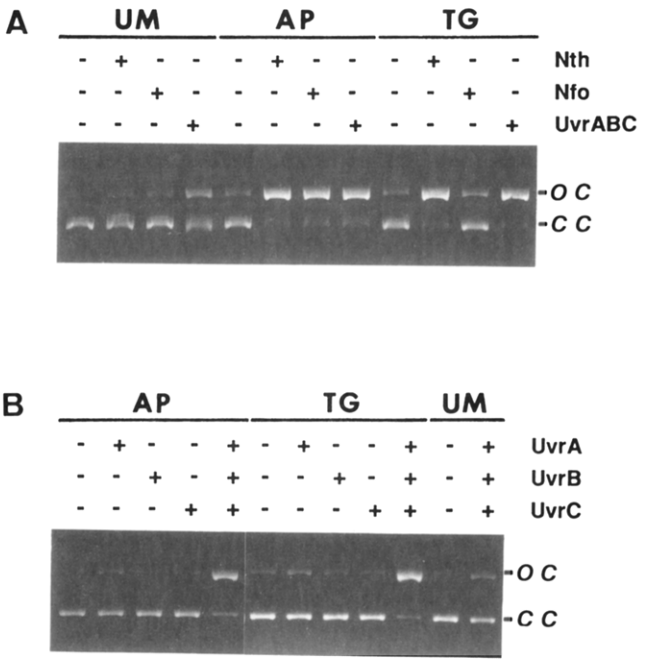

FIGURE 1: (A) Incision of TG-DNA and AP-DNA by (A)BC excinuclease. pBR322 (0.2 μg) containing about 1.4 AP site or TG per molecule was incubated in 25 μL of ABC excinuclease buffer with the indicated enzymes for 30 min and then analyzed by agarose gel electrophoresis. The reaction mixtures contained 0.2 nM endonuclease III (Nth), 0.4 nM endonuclease IV (Nfo), or 8 nM UvrA + 180 nM UvrB + 20 nM UvrC (UvrABC) as indicated. UM, unmodified DNA; CC, covalently closed circles; OC, open circles of plasmid. (B) Effect of ABC excinuclease subunits on "undamaged" AP- and TG-DNA. pBR322 DNA (0.2 μg) not subjected to a modifying treatment or heated at low pH (AP-DNA) or treated with 0.04% OsO_4 (TG-DNA) was incubated with UvrA (8 nM), UvrB (180 nM), and UvrC (20 nM) at 37 °C for 30 min in 25 μL of reaction buffer and then analyzed on 1% agarose gel.

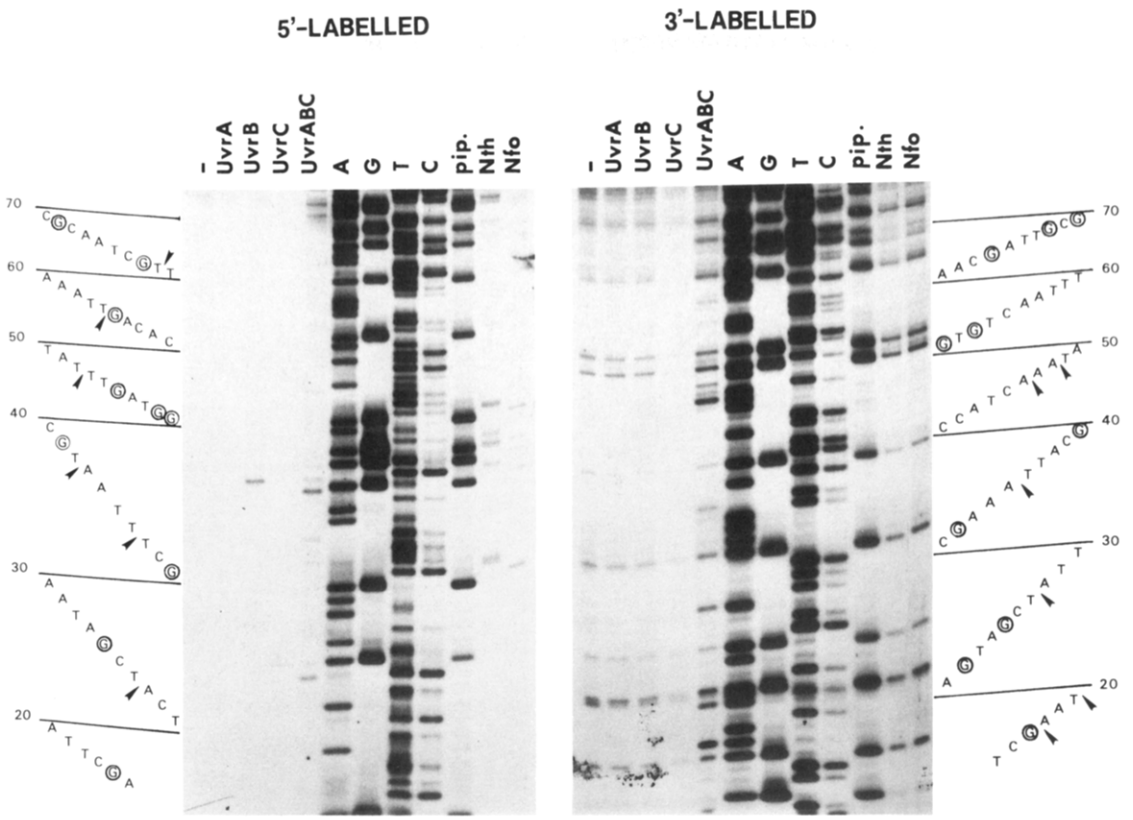

FIGURE 2: Sites of incision of AP-DNA by repair enzymes. The $(Eco\text{RI}–Hae\text{III})_{174}$ fragment of pBR322 either 5′ or 3′ labeled at the *Eco*RI site was depurinated and then treated with the indicated enzymes or with piperidine and analyzed on 8% sequencing gels. The circles indicate the missing base, and the arrows point to the sites of incision of (A)BC excinuclease. The source of the unique incision with UvrB subunit is unknown; the high background in the ABC lane is probably due to a contaminating exonuclease and the bands seen at positions of Gs in the undamaged DNA to background depurinitation during preparation of the fragment. Note that only Gs were depurinated to a significant extent. The bases are numbered according to the standard pBR322 numbering system. Pip, DNA treated with piperidine. A, G, T, and C correspond to Maxam–Gilbert reactions for A + G, G, T + C, and C, respectively. The apparent greater cleavage 3′ to the AP sites than cleavage 5′ is not real and is caused by the higher amount of substrate used in 3′ incision experiments.

the sample at 90 °C in 1 M piperidine for 30 min.

Results

Incision of TG-DNA and AP-DNA by ABC Excinuclease. Figure 1A shows the effect of (A)BC excinuclease on plasmid DNA containing either thymine glycols or AP sites. The enzyme nicks DNA containing either lesion. As positive controls we used *E. coli* endonucleases III (Nth) and IV (Nfo) in these incision experiments. Endonuclease III is an iron–sulfur protein (Cunningham et al., 1989) that has thymine glycol glycosylase–AP endonuclease function (Demple & Linn, 1980). It releases the thymine glycol and then breaks the phosphodiester bond 3′ to the AP site by β-elimination (Bailly & Verly, 1987; Kow & Wallace, 1987; Kim & Linn, 1988). It also acts on simple AP sites, breaking the phosphodiester bond 3′ to the abasic sugar [type I AP endonuclease; see Sancar and Sancar (1988)]. Accordingly, plasmid DNA containing either thymine glycols or AP sites is nicked by endonuclease III. Endonuclease IV is a type II AP endonuclease that incises 5′ to AP sites but has no glycosylase function (Ljungquist, 1977; Cunningham et al., 1986; Levin et al., 1988). As expected, this enzyme does not nick TG-DNA but nicks AP-DNA.

Requirement for All Three Subunits of ABC Excinuclease. It is well-known that many basic proteins with no possible function in DNA repair (e.g., cytochrome *c*) promote cleavage of AP sites presumably by β-elimination [see Friedberg (1985)]. Therefore, it is important to show the specificity of nicking of such sites by an enzyme in order to claim with any certainty that that enzyme acts on AP sites. (A)BC excinuclease requires the participation of all three subunits and ATP to carry out its particular mode of incision (Sancar & Rupp, 1983). Therefore, to demonstrate that the observed effect of the enzyme was a property of the (A)BC excinuclease enzyme complex and was not due to contaminating glycosylases and AP endonucleases in the enzyme preparation, we conducted the incision reactions with individual subunits and with combinations of all three. The results are shown in Figure 1B, and a quantitative evaluation of these results is presented in Table I. The UvrA subunit has some AP endonuclease contamination, presumably endonuclease III because it also acts on TG-DNA. The UvrB and UvrC subunits are free of thymine glycol glycosylase and AP endonucleases. In calculating the specific thymine glycol or AP site induced ABC excinuclease incision, however, one needs to consider an additional source of background: the mixture of UvrA, UvrB, and UvrC causes some nicking of even "undamaged" DNA

Table I: Effect of (A)BC Excinuclease on AP-DNA and TG-DNA[a]

enzyme	no. of nicks/plasmid		
	UM-DNA	AP-DNA	TG-DNA
UvrA	0.04	0.11	0.06
UvrB	0.01	0.00	0.00
UvrC	0.00	0.03	0.01
UvrABC	0.22	1.06	1.27
Nth	0.03	1.30	1.26
Nfo	0.02	1.08	0.08

[a] Both AP-DNA and TG-DNA contained about 1.4 AP sites or TG, respectively, per plasmid molecule as determined from limit digestions with Nfo and Nth, respectively. UM-DNA, unmodified (nondamaged) DNA.

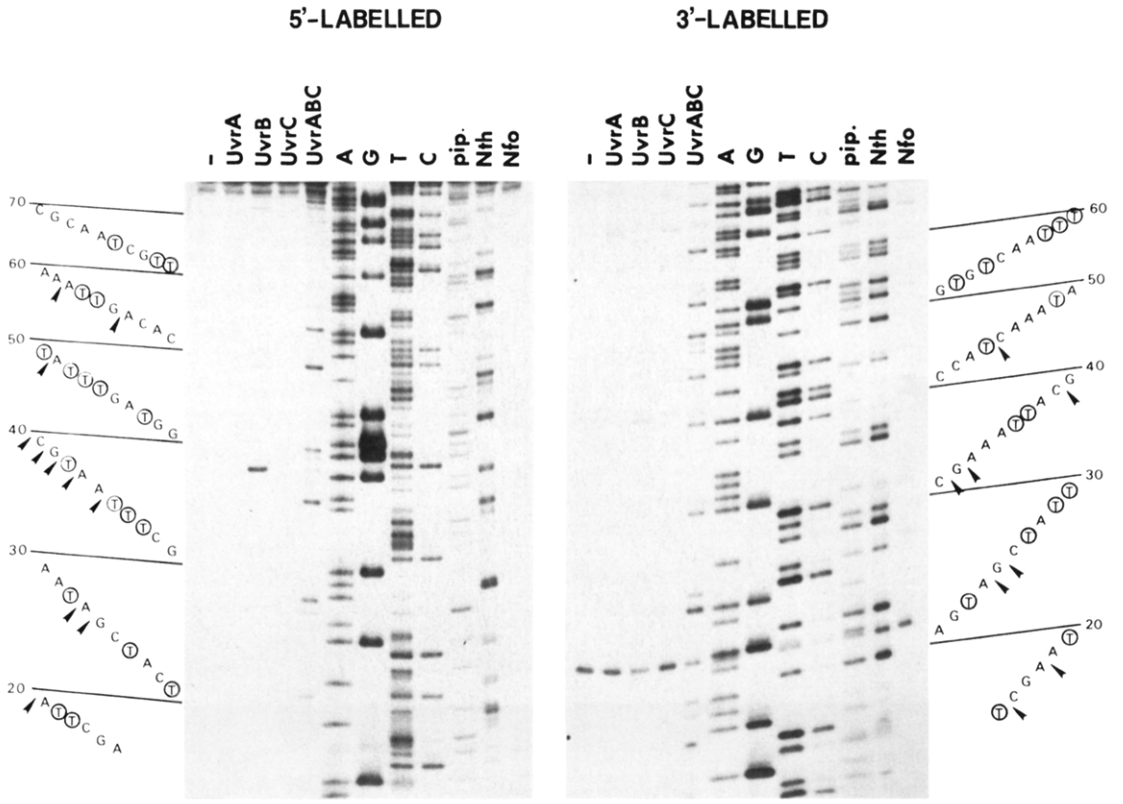

FIGURE 3: Sites of incision of TG-DNA by repair enzymes. The $(EcoRI–HaeIII)_{174}$ fragment labeled at the *Eco*RI terminus was oxidized with OsO_4, and then about 3000 cpm of labeled DNA was incubated with the indicated enzymes or piperidine (Pip) and analyzed on 8% sequencing gels. The circles indicate the modified bases, and the arrows point to the incision sites of ABC excinuclease.

(Van Houten & Sancar, 1987; Caron & Grossman, 1988), and therefore this must be subtracted from the nicks introduced into AP-DNA and TG-DNA to obtain the value for incisions by ABC excinuclease that is specific for these two latter lesions. When the corrections are made, it is apparent that the excinuclease (at relatively higher concentrations of its subunits compared to the endonucleases) acts efficiently on AP sites and thymine glycols as evidenced by the number of specific nicks made by (A)BC excinuclease compared to the number of nicks made by endonuclease IV and endonuclease III, respectively (Table I).

Mode of Incision of TG-DNA and AP-DNA by (A)BC Excinuclease. It was of interest to determine whether (A)BC excinuclease incised AP-DNA and TG-DNA at the site of lesion (AP endonuclease) or incised on both sides of the lesion at a distance in the usual fashion (excinuclease). We used terminally labeled DNA for this purpose and obtained the results shown in Figures 2 and 3. While endonuclease IV and endonuclease III incised DNA at the sites of AP and TG, respectively, (A)BC excinuclease incised the eighth phosphodiester bond 5′ and the fifth phosphodiester bond 3′ to both lesions. Thus, the (A)BC excinuclease eliminates these lesions from DNA by the same mechanism as it removes bulky base adducts. It is interesting to note, however, that while (A)BC excinuclease incised TG- or AP-containing plasmid DNA to the same extent as the AP endonucleases (Figure 1 and Table I), it was less effective on these lesions in linear DNA compared to the AP endonucleases. This may be taken as an indication for the preference of the (A)BC excinuclease for superhelical DNA although other explanations are also possible.

Functional Overlap of Base Excision and Nucleotide Excision. Nucleotide excision repair in *E. coli* is relatively cumbersome: UvrA delivers UvrB to the damage site and then dissociates from the UvrB–DNA complex. This UvrB–DNA complex is recognized by UvrC and the two incisions are made (Orren & Sancar, 1989). The repair reaction is quite slow, proceeding at a rate of 0.24 adduct/min in vivo and about 0.1 adduct/min in vitro under turnover conditions (Husain et al., 1985). Furthermore, there are about 10 UvrA dimers (functional form) and about 250 UvrB molecules in an uninduced *E. coli* [see Sancar and Sancar (1988)]. It is therefore conceivable that 10 UvrA dimers would deliver 250 UvrB onto AP sites or TG sites and (since there are only about 10 UvrC molecules per cell) these UvrB–DNA complexes would block access to the more economic and efficient TG-glycosylase–AP endonuclease and/or AP endonucleases. We, therefore, wished to know whether specific UvrA–DNA or, more importantly, the very stable UvrB–DNA complexes interfered with endonuclease III or endonuclease IV. We incubated plasmid DNA containing either TG or AP sites with UvrA, UvrB, or UvrA and UvrB, then added either UvrC or endonucleases III or IV, and followed the reaction kinetics. The results are shown in Figure 4. As expected, addition of UvrC resulted in nicking of DNA by (A)BC excinuclease. Neither UvrA nor UvrA plus UvrB had an effect on the reaction kinetics of the endonucleases III or IV. We have shown that the concentrations of the subunits used in these experiments are sufficient to protect the lesion from DNase I (Van Houten et al., 1988) in the case of UvrA and to saturate all the AP or TG sites in the case of UvrB (Orren & Sancar, 1989). We therefore conclude that binding of the subunits of (A)BC excinuclease does not interfere with binding of, and repair by, enzymes involved in base excision repair and that the two pathways may work in a complementary and cooperative fashion.

Discussion

Active oxygen species generated as byproducts of oxidative

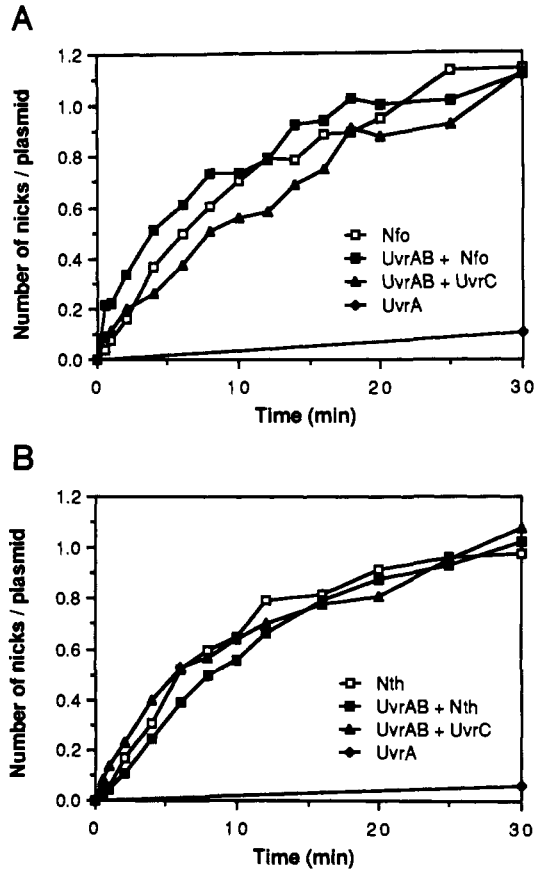

FIGURE 4: Kinetics of incision of AP- and TG-DNAs by endonucleases III and IV in the presence of UvrA and/or UvrB subunits of (A)BC excinuclease. pBR322 DNA (2.0 μg) was preincubated with UvrA (8 nM) and/or UvrB (180 nM) for 15 min at 37 °C in 200 μL of reaction mixture. At "zero time" UvrC (20 nM), endonuclease III (0.2 nM), or endonuclease IV (0.4 nM) was added, 12.5-μL samples were taken at time intervals, the reaction was stopped with 0.05% sodium dodecyl sulfate, and then the products were separated on 1% agarose gels. From the amount of superhelical DNA remaining the number of incisions per plasmid was calculated and plotted.

metabolism or by ionizing radiation are perhaps the most common DNA-damaging chemical species. It is not surprising, therefore, that cells have inducible enzymes that deactivate these species (Hassan & Fridovich, 1977, 1978) and repair oxidative DNA damage (Demple & Halbrook, 1983). The discovery in enteric bacteria of several regulons that comprise 60–70 proteins induced by oxidative stress (Chrisman et al., 1985; Kogoma et al., 1988; Walker & Kogoma, 1989; Greenberg & Demple, 1989) is indicative of the importance of oxidative DNA damage and the considerable cellular resources that are mobilized to overcome the oxidative stress. It is therefore fitting that the (A)BC excinuclease, a very versatile enzyme with wide substrate range, is also utilized to repair DNA damage by oxidizing agents. However, two interrelated observations have been generally taken as evidence that the enzyme was not involved in repairing oxidative damage. First, cells defective in this enzyme are sensitive to agents that make bulky DNA adducts (Howard-Flanders & Boyce, 1966), such as psoralen and mitomycin C, and second, these mutants are not sensitive to ionizing radiation, methylating agents (Howard-Flanders & Boyce, 1966), or H_2O_2 (Imlay & Linn, 1986).

More recent experiments with *E. coli* strains with double and triple mutations have shed some light on this apparent paradox. *E. coli* mutants defective in O^6-mGua DNA methyltransferase have increased sensitivity to MNNG when they are also defective in (A)BC excinuclease (Van Houten & Sancar, 1987; Samson et al., 1988; Rossi et al., 1989). Furthermore, it has been shown that the purified enzyme excises O^6-mGuA from DNA (Voigt et al., 1989). Similarly, Saporito et al. (1989) were able to construct *xth nfo*, *xth uvrA*, *nfo uvrA* double mutants but not the *xth nfo uvrA* triple mutant, suggesting a functional redundancy of the three proteins which is probably the removal of AP sites, although alternative explanations are also possible.

The recent genetic experiments mentioned above as well as the biochemical results presented in this paper suggest that while the biochemical mechanisms defining the various repair pathways are clearly distinct mechanistically, there is considerable functional overlap between nucleotide excision repair and direct repair by methyl transferases, as well as base excision repair by glycosylases and AP endonucleases. Clearly, while it is useful to think of these mechanisms as separate biochemical pathways, there must be considerable overlap and cooperation between these pathways even at the molecular level. This is likely to be the case with mammalian excision nuclease(s) as well, because rats excrete thymidine glycol and thymine glycol in their urine (Catchart et al., 1984), which are presumably released from DNA by an excision nuclease and a DNA glycosylase, respectively.

Finally, the observations reported in this paper bring into focus once more the substrate specificity of (A)BC excinuclease [see Voigt et al. (1989)]. Two of the well-investigated substrates, the thymine dimer and the *cis*-Pt–GG diadduct, induce a 30–40° bend into the major groove (Pearlman et al., 1985; Husain et al., 1988; Rice et al., 1988) while a psoralen cross-link appears to bend DNA by some assays (Tomic et al., 1987; Shi et al., 1988) but not by others (Haran & Crothers, 1988). Structural studies on O^6-mGua, thymine glycol, or AP site containing DNA have not revealed any significant bend or gross structural perturbation in the duplex (Patel et al., 1987; Clark et al., 1987; Kalnik et al., 1989) containing these modifications. On the other hand, one to three nucleotide loops that do cause bending (Hsieh & Griffith, 1989) are not substrates for the enzyme (Thomas et al., 1986). So far, all modifications that involve covalent changes in DNA have been found to be substrates. A reasonable model for recognition and repair would be that structural perturbations which make $(UvrA)_2$ unload UvrB onto DNA are repaired by (A)BC excinuclease. This way of thinking about the problem shifts the question of damage recognition from a static one to a kinetic or a dynamic one. We have now a simple method for investigating the loading process (Orren & Sancar, 1989), and experiments along these lines may help us understand what makes UvrA unload UvrB and thus define the substrate specificity of (A)BC excinuclease.

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