Utilization of DNA photolyase, pyrimidine dimer endonucleases, and alkali hydrolysis in the analysis of aberrant ABC excinuclease incisions adjacent to UV-induced DNA photoproducts

Gary M.Myles, Bennett Van Houten and Aziz Sancar

University of North Carolina, School of Medicine, Department of Biochemistry 231H, Chapel Hill, NC 27514, USA

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ABSTRACT

ABC excinuclease of *Escherichia coli* removes 6-4 photoproducts and pyrimidine dimers from DNA by making two single strand incisions, one 8 phosphodiester bonds 5' and another 4 or 5 phosphodiester bonds 3' to the lesion. We describe in this communication a method, which utilizes DNA photolyase from *E. coli*, pyrimidine dimer endonucleases from *M. luteus* and bacteriophage T4, and alkali hydrolysis, for analyzing the ABC excinuclease incision pattern corresponding to each of these photoproducts in a DNA fragment. On occasion, ABC excinuclease does not incise DNA exclusively 8 phosphodiester bonds 5' or 4 or 5 phosphodiester bonds 3' to the photoproduct. Both the nature of the adduct (6-4 photoproduct or pyrimidine dimer) and the sequence of neighboring nucleotides influence the incision pattern of ABC excinuclease. We show directly that photolyase stimulates the removal of pyrimidine dimers (but not 6-4 photoproducts) by the excinuclease. Also, photolyase does not repair CC pyrimidine dimers efficiently while it does repair TT or TC pyrimidine dimers.

INTRODUCTION

ABC excinuclease is an ATP dependent DNA repair enzyme composed of three subunits, the UvrA ($M_r = 103,874$), UvrB ($M_r = 76,118$), and UvrC ($M_r = 66,038$) proteins (reviewed in 1, 2-4). Using DNA fragments randomly modified with ultraviolet light, it has been concluded that the enzyme hydrolyzes the 8th phosphodiester bond 5' and the 4th or 5th phosphodiester bond 3' to UV induced pyrimidine dimers and 6-4 photoproducts (5,6). Because the ABC excinuclease incision pattern for both of these photoproducts appears to be identical, it has been impossible to study directly the relative contribution of each photoproduct to the 5' and 3' incisions. Using DNA photolyase, we have extended the work of Lippke et al. (7) by designing a method for determining the approximate frequency of formation of each type of photoproduct at a specific pair of nucleotides on a DNA fragment. This method takes advantage of the pyrimidine dimer specificity of both *E. coli* DNA photolyase and *M. luteus* and T4-phage pyrimidine dimer endonucleases as well of the susceptibility of 6-4 photoproducts to alkali hydrolysis.

Briefly, if two adjacent nucleotides yield exclusively pyrimidine dimers upon UV irradiation, then the adduct will be repaired completely by DNA photolyase and therefore will no longer be susceptible to incision by ABC excinuclease. Also, such a DNA adduct will be incised by a pyrimidine dimer specific endonuclease. In contrast, adjacent nucleotides yielding UV induced 6-4 photoproducts will be hydrolyzed by piperidine but not repaired by photolyase nor incised by a pyrimidine dimer specific endonuclease. If the two nucleotides have a propensity for formation of each photoproduct, the fraction of each photoproduct contributing to the 5' and 3' incision can be elucidated by reacting the DNA with an appropriate combination of repair enzymes and alkali hydrolysis. We describe in some detail the application of this method to the analysis of non-standard incisions made by ABC excinuclease.

Analysis of the digestion products of damaged DNA on sequencing gels reveals bands that are not consistent with the general pattern of ABC excinuclease incision. Of particular importance is the observation that, with 5' labeled DNA, bands appear which correspond to cutting at the 7th instead of the 8th phosphodiester bond (7 nucleotides) 5' to the DNA adduct (5, 8). In this study, we have investigated the apparent plasticity in the ABC excinuclease cleavage specificity with regard to hydrolysis of phosphodiester bonds both 5' and 3' to DNA damage. We have used the method described above to address the question of whether this flexibility in the cutting pattern is caused by the type of UV induced DNA adduct or the nucleotide sequence surrounding the adduct and hence the local DNA structure.

MATERIALS AND METHODS

Materials.

Restriction enzymes, bacterial alkaline phosphatase, polynucleotide kinase and DNA polymerase I (Klenow fragment) were purchased from New England BioLabs, Bethesda Research Laboratory and Boehringer Mannheim Biochemicals, radioisotopes were obtained from Amersham and ICN.

Enzymes.

The subunits of ABC excinuclease were purified separately and the enzyme was reconstituted from the individually purified subunits (9). *E. coli* DNA photolyase was purified as described previously (10). *M. luteus* UV endonuclease was a generous gift of Dr. P. Doetsch (Emory University) and T4-phage endonuclease V (endo V) was kindly provided by Dr. D. Brash (NIH).

Substrates.

The substrates for these studies were restriction fragments from pUNC 1986 (a plasmid carrying the *uvrA* gene) (2). Specifically, the fragment for experiments on 5' ABC excinuclease incisions spanned 198 nucleotides from the *HinF* I site at position 1882 bp to the *Nru* I site at 2080 bp. This fragment was prepared by first digesting the plasmid at positions 1870 and 2080 with *Mlu* I and *Nru* I, respectively. This 210 bp fragment was purified by electrophoresis on a preparative 8% polyacrylamide gel. After removing the terminal 5' phosphates with bacterial alkaline phosphatase (BRL), the fragment was labeled on the 5' termini with T-4 kinase (BRL) and [γ - 32 P]-ATP (ICN) by standard means. Digestion of the *Mlu* I₍₁₈₇₀₎ - *Nru* I₍₂₀₈₀₎ fragment with *HinF* I yielded a 198 bp fragment uniquely labeled at the 5' terminus at the *Nru* I site. For investigation of the 3' cleavage site, the 102 bp fragment from an *Nci* I site at position 1978 to an *Nru* I site at position 2080 was used. This fragment was prepared by digesting pUNC 1986 with *Nci* I and the 184 bp fragment was purified as before. After removal of the 3' phosphates with bacterial alkaline phosphatase, the 3' termini were labeled with [α - 32 P]dCTP (Amersham) and the Klenow fragment of DNA polymerase I (Boehringer-Mannheim). Digestion with *Nru* I yielded a 102 bp fragment uniquely 3' labeled at the *Nci* I site, position 1978. Both of these fragments overlap in the region of staggered ABC excinuclease incisions described in this communication. The terminally labeled DNA fragment, 10 μ g/ml, was irradiated in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 1 mM EDTA, with 4000 J/m² of 254 nm light from a germicidal lamp.

Uniformly labeled DNA was prepared by the primer extension method using M13mp7 template, the commercially available primer (Bethesda Research Laboratory) and all four dNTPs of which the dATP contained 32 P at the α position. The radiolabeled M13mp7 was separated from unincorporated 32 P-dATP by electrophoresis on a 5% polyacrylamide gel. The full length DNA band was sliced from the gel, electroeluted, and dialyzed against 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 1 mM EDTA.

Enzymatic assays and analysis of the reaction products.

Enzymatic treatments with photolyase, T4-phage and *M. luteus* endonucleases as well as ABC excinuclease were conducted in ABC excinuclease buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 10 mM DTT, 2 mM ATP and 100 μ g/ml bovine serum albumin. The reaction mixture (50 μ l) contained 0.2-0.4 pmol of terminally-labeled, UV-irradiated DNA (approximately 3 adducts per molecule for a total of 0.6-1.2 pmol photoproducts)

and, when indicated, 20 pmol of photolyase, 1 unit of T4-phage or *M. luteus* endonuclease, and 1 pmol each of the subunits (UvrA, UvrB and UvrC) of ABC excinuclease. Samples treated with photolyase were incubated in the dark for 15 minutes at 23°C prior to 60 minute illumination from a Black Light ($\sim 1000 \mu\text{Wcm}^{-2}$). Photolyase was removed from the photoreactivated DNA by phenol extraction and ethanol precipitation prior to digestion with either ABC excinuclease, *M. luteus* UV endonuclease, or endo V. Digestions with the ABC excinuclease and endonucleases were accomplished by incubating the reaction mixtures at 37°C for 15 min. The reaction products were then processed and analyzed on DNA sequencing gels (1) as described previously (5). Hot alkali digestion was accomplished by heating the DNA at 90°C for 30 min in 0.5 M piperidine (12,13).

M. luteus UV endonuclease and endo V have identical enzymatic activities, that is, they both cleave the N-glycosidic bond of the 5' nucleotide of a pyrimidine dimer and hydrolyse the phosphodiester bond 3' to the resultant apyrimidinic site. These enzymes can, therefore, be used interchangeably to quantitate pyrimidine dimer formation in UV irradiated DNA. In this study, where indicated, the 5' labeled DNA fragment was digested with *M. luteus* UV endonuclease and the 3' labeled DNA fragment was digested with endo V.

Quantitation of the extent of product formation was accomplished by densitometric scanning of the autoradiograms. For this purpose we used an optonics P-1000 film scanner with an AED graphics terminal. Data analysis was performed on a VACS 11/730 computer using the algorithm, Gelscan, developed by Frank Hage of the protein crystallography facility at the University of North Carolina.

RESULTS

Use of various DNA repair enzymes and alkali hydrolysis to analyze photo-product distribution in UV irradiated DNA.

The relative frequency at which a pair of nucleotides in a DNA fragment form 6-4 photoproducts and pyrimidine dimers upon UV irradiation can be determined by taking advantage of the pyrimidine dimer specificity of DNA photolyase, *M. luteus* UV endonuclease, and endo V (12, 14-16) as well as the susceptibility of 6-4 photoproducts to alkali hydrolysis (7,17). A fragment of DNA irradiated with UV light and treated with ABC excinuclease yields a unique series of bands corresponding to cleavage 8 phosphodiester bonds (for 5' labelled DNA) or 4 or 5 phosphodiester bonds (for 3' labelled DNA) from

the DNA adduct (Fig. 1, lanes 2 and 10; Fig. 3, lanes 2 and 11). Incubation of a UV irradiated DNA fragment with DNA photolyase (in the dark) prior to incision by ABC excinuclease does not result in an altered cleavage pattern although certain bands appear darker in the presence of photolyase (Fig. 1b, lane 3). If the same fragment of DNA that is incubated with photolyase is irradiated with 366 nm light such that pyrimidine dimers are removed prior to ABC excinuclease incision, a modified band pattern is revealed - some bands staying the same intensity, some decreasing in intensity, and others disappearing altogether. These bands reveal lesions which are photolyase resistant, fractionally photolyase resistant, and photolyase sensitive, respectively. Thus, by photoreversing pyrimidine dimers prior to ABC excinuclease incision, the distribution of pyrimidine dimers and the other DNA adducts (presumably 6-4 photoproducts) is revealed.

Confirmation of the distribution and relative abundance of pyrimidine dimers along a UV irradiated DNA fragment can be achieved by substituting a pyrimidine dimer specific endonuclease, either *M. luteus* UV endonuclease or endo V, for ABC excinuclease in the previous analysis (Fig. 1, lanes 9-12). Photoreversal prior to endonuclease treatment confirms the identity of a DNA adduct being pyrimidine dimer. It should be pointed out, however, that CC pyrimidine dimers, while recognized by both pyrimidine dimer endonucleases, are not efficiently reversed by DNA photolyase (Fig. 1, lane 12).

That non-pyrimidine dimer UV photoproducts are in fact 6-4 photoproducts can be demonstrated by alkali hydrolysis of UV-irradiated DNA following photoreactivation of pyrimidine dimers with photolyase (Fig. 2, lane 8). Lippke et al. (7) have shown that piperidine specifically hydrolyzes DNA at the site of 6-4 photoproducts. We have taken advantage of this observation in conjunction with the known pyrimidine dimer specificity of *M. luteus* UV endonuclease and endo V to elucidate the photoproduct distribution in UV irradiated DNA (Fig. 2, lanes 7 and 8; Fig. 3, lanes 9 and 10). Figures 2 and 3 (lanes 9 and 11, respectively) also show that the ABC excinuclease incision pattern corresponds approximately to the sum of the band patterns for the endonuclease and piperidine treated UV irradiated DNA. Thus, ABC excinuclease has a general photoproduct specificity - recognizing both pyrimidine dimers and 6-4 photoproducts.

Relative contributions of pyrimidine dimers and 6-4 photoproducts to incisions by ABC excinuclease.

The broad UV photoproduct specificity of ABC excinuclease is most evident from the intensity of the incision bands corresponding to hydrolysis

of the phosphodiester bond at T₂₆C₂₇ and C₂₇G₂₈ which are caused by the photoproducts at T₃₄T₃₅ and T₃₅C₃₆, respectively. Analysis of the photoproduct composition by *M. luteus* UV endonuclease and hot alkali digestion indicate that > 90% of the photoproduct at T₃₄T₃₅ is pyrimidine dimer while about 90% of the photoproduct at T₃₅C₃₆ is 6-4 photoproduct (Figure 2, lanes 7 and 8). The intensity of the ABC excinuclease band (at T₂₆C₂₇), corresponding to the removal of the pyrimidine dimer at T₃₄T₃₅, indicates that about 10-15% of the dimer at this site is removed while the intensity of the band at C₂₇G₂₈ indicates that all of the photoproducts at this site (~ 90% 6-4 photoproduct and 10% pyrimidine dimer) are removed by the excinuclease.

Figure 1. Incision of UV-irradiated DNA by ABC excinuclease and *M. luteus* UV endonuclease in the presence of *E. coli* DNA photolyase. The DNA fragment was 5' labeled at the Nru I site, UV irradiated, treated with various enzymes, as indicated, and then analyzed on DNA sequencing gels. Equal amounts of DNA were loaded into each well. A, short run (2 hrs); B, long run (4 hrs). Lane 1, non-irradiated DNA plus ABC excinuclease. Lane 2, UV-irradiated DNA plus ABC excinuclease; Lane 3, UV-irradiated DNA plus photolyase (dark) plus ABC excinuclease; lane 4, UV-irradiated DNA plus photolyase (60 min photoreactivating light) plus ABC excinuclease; Lanes 5-8, A + G, G, T + C, and C ladders of Maxam and Gilbert sequence; Lane 9, non-irradiated DNA plus *M. luteus* endonuclease; Lane 10, UV-irradiated DNA plus *M. luteus* UV endonuclease; Lane 11, UV-irradiated DNA plus photolyase (dark) plus *M. luteus* UV endonuclease; Lane 12, DNA plus photolyase (60 min photoreactivating light) plus *M. luteus*. The background bands seen in lanes 1 and 9 were also present in untreated DNA (data not shown) and are presumably due to depurination and/or depyrimidination induced at specific sites during handling of DNA.

Figure 2. Contributions of pyrimidine dimers and 6-4 photoproducts to incisions by ABC excinuclease. DNA labeled at the 5' Nru I terminus was subjected to various treatments and then analyzed on an 8% DNA sequencing gel. Lane 1, non-irradiated DNA plus ABC excinuclease; Lane 2, non-irradiated DNA plus piperidine; Lanes 3-6, A + G, G + G, T + C, and C; Lane 7, UV-irradiated DNA plus *M. luteus* UV endonuclease; Lane 8, UV-irradiated DNA photoreactivated and hydrolyzed with piperidine; Lane 9, UV-irradiated DNA plus ABC excinuclease. Note that the *M. luteus* UV endonuclease bands migrate between the two pyrimidines that make up the corresponding pyrimidine dimer while the piperidine generated bands migrate about 1 space slower than the 3' pyrimidine band of the 6-4 photoproduct (Lippke et al., 1981). The "normal" ABC excinuclease bands migrate 6.5 spaces faster than the 5' pyrimidine bands of the corresponding photoproducts (both pyrimidine dimers and 6-4 photoproducts).

Figure 3. Digestion of 3'-labeled, UV-irradiated DNA by ABC excinuclease, T4-phage endonuclease V, and hot alkali. The DNA was labeled at the 3' terminus of the Nci I site and analyzed on an 8% sequencing gel. Lane 1, non-irradiated DNA plus ABC excinuclease; Lane 2, UV-irradiated DNA plus ABC excinuclease; Lane 3, UV-irradiated DNA plus photolyase (dark) plus ABC excinuclease; Lane 4, UV-irradiated DNA plus photolyase (60 min photoreactivating light) plus ABC excinuclease; Lanes 5-8, A + G, G, T + C and C sequence reactions; Lane 9, UV-irradiated DNA plus T4 endonuclease V; Lane 10, UV-irradiated DNA hydrolyzed with piperidine; Lane 11, UV-irradiated DNA digested with ABC excinuclease. Note that the T4 endonuclease V bands migrate one space slower than the 5' pyrimidine of the corresponding dimers, the hot alkali bands migrate with the 3' pyrimidine of the 6-4 photoproduct, and the "normal" ABC excinuclease bands migrate 3 or 4 spaces 3' to the 3' pyrimidine of the dipyrimidine making either photoproduct. The particular T4 endonuclease V used in this experiment contained some non-specific endonuclease which produced extraneous bands that do not fit the pattern. Also note that while the ABC excinuclease digestion in Lanes 2-4 is partial, the one in lane 11 is complete. Lanes 1-4 and 9-11 are from two separate experiments.

A close examination of the autoradiogram reveals this general pattern; wherever there is a pyrimidine dimer or a 6-4 photoproduct (as indicated by the *M. luteus* UV endonuclease cleavage or alkali hydrolysis, respectively), there is a corresponding incision by ABC excinuclease 7 nucleotides 5' to the photoproduct.

Staggered incision by ABC excinuclease on the 5' side of photoproducts.

We previously reported that while ABC excinuclease hydrolyzes the 4th and 5th phosphodiester bonds 3' to pyrimidine dimers with about equal frequency, it incises exclusively the 8th phosphodiester bond 5' to the dimers. Analysis of a large number of incision sites on DNA sequencing gels revealed exceptions to this rule. In Figure 1 we present results that indicate that even though the majority of incisions made by ABC excinuclease are at the 8th phosphodiester bond 5' to potential UV induced photoproducts, there are two examples of incisions at alternate sites: the incisions produced at A₃₇A₃₈, A₃₈C₃₉ and C₃₉T₄₀ are presumably caused by ABC excinuclease acting on the photoproduct at T₄₅T₄₆ and the incisions made at G₆₂T₆₃ and T₆₃G₆₄ result from the photoproduct at T₇₀T₇₁ (Fig. 1, lane 2). All other incisions produced by ABC excinuclease in this fragment are 7 nucleotides 5' to a pyrimidine-pyrimidine sequence and are thus consistent with hydrolysis of the 8th phosphodiester bond 5' to a pyrimidine dimer or a 6-4 photoproduct.

The cause of staggered incisions 5' to UV photoproducts.

To determine whether the staggering caused by the photoproducts at T₄₅T₄₆ and T₇₀T₇₁ were due to pyrimidine dimers, 6-4 photoproducts or both adducts, we examined the ABC excinuclease incision pattern after treatment of this fragment with UV light and *E. coli* DNA photolyase. Figure 1 (lanes 3 and 4) demonstrates the effect of photolyase and photolyase plus photoreactivating light, respectively, on the incision activity of ABC excinuclease. Densitometric scanning of autoradiograms, exposed to the radioactive gel for a shorter period of time, indicated that photolyase stimulates the ABC excinuclease activity at specific sites when the photolyase-DNA mixture is incubated with the nuclease in the absence of photoreactivating light (data not shown). This is seen as an enhancement in the intensity of ABC excinuclease generated bands in lane 3. (this is most evident in Figure 1b). This enhancement is particularly clear at the bands corresponding to incisions at the G₆₂T₆₃ and A₇₆G₇₇ phosphodiester bonds. These results suggest that the enhancement of the excinuclease activity by photolyase is dimer-specific. Lane 4 contains DNA photoreactivated prior to the excinuclease treatment. Although the intensity of the bands in these lanes is considerably lower than that in lane 3, the similarity in band patterns between the two lanes is striking. This similarity in incision pattern is consistent with two interpretations: either photolyase repaired only a fraction of the dimers at each pyrimidine-pyrimidine site or, alternatively,

at each of these sites, in addition to dimers, other photoproducts (presumably 6-4 photoproducts) were formed and not repaired by DNA photolyase. To differentiate between these two possibilities, we treated the irradiated DNA fragment with *M. luteus* UV endonuclease, which is specific for pyrimidine dimers. Figure 1 (lane 10) shows DNA digested to completion with the endonuclease. The band pattern in this lane reveals not only the location of pyrimidine dimers but also the extent of dimer formation at each site. When the endonuclease digestion is conducted in the presence of photolyase, without photoreactivating light, some inhibition of the endonuclease is observed (lane 11). This result is in agreement with earlier observations (18) and is consistent with an overlap in the DNA binding sites for photolyase and *M. luteus* endonuclease. If the DNA fragment is incubated with DNA photolyase in the presence of photoreactivating light prior to treatment with *M. luteus* endonuclease (lane 12), almost all of the endonuclease sensitive sites (with the exception of those at a CC sequence) are eliminated, demonstrating that photoreactivation of the pyrimidine dimers is complete. Thus, we conclude that the ABC excinuclease incisions of photoreactivated DNA are not caused by pyrimidine dimers but by other photoproducts. Alkali hydrolysis of the photoreactivated DNA strongly suggests that these non-dimer adducts are 6-4 photoproducts (see figure 2) (7).

Having established the identity of the UV photoproducts, we can now answer the question of which photoproducts are responsible for staggering. The adduct at T₄₅T₄₆ is completely eliminated by photoreactivation as determined by both ABC excinuclease and *M. luteus* UV endonuclease digestion and, therefore, must be a pyrimidine dimer (Fig. 1b, lanes 4 and 12, respectively). This dimer results in incisions at the 6th, 7th and 8th phosphodiester bonds 5' to the DNA adduct. In contrast, incision at the G₆₂T₆₃ and T₆₃G₆₄ phosphodiester bonds seems to result from the 6-4 photoproduct at T₇₀T₇₁: the intensity of the band corresponding to the distal incision is enhanced by photolyase in the dark but the intensity of the proximal incision is not influenced (Fig. 1, lane 3). Following photoreactivation, all of the pyrimidine dimers are eliminated from this site as determined by *M. luteus* UV endonuclease digestion, but photoreactivation does not eliminate the staggered ABC excinuclease cuts, rather it changes their relative frequencies. The excinuclease incision at the G₆₂T₆₃ phosphodiester bond, which is enhanced by photolyase in the dark, is reduced in intensity (but not eliminated), following photoreactivation suggesting that ABC excinuclease mediated incision at this site is primarily due to the pyrimidine dimer and partially

to 6-4 photoproduct. The nuclease incisions at T₆₃G₆₄ are essentially unchanged by preincubating the fragment with photolyase either in the dark or with photoreactivating light and, therefore, are caused by a 6-4 photoproduct. While a pyrimidine dimer at T₇₀T₇₁ is removed by ABC excinuclease by incision of the 8th phosphodiester bond, a 6-4 photoproduct at this site causes incision by the enzyme either at the 7th or 8th phosphodiester bond. In summary, these data suggest that staggering in the 5' cleavage pattern of ABC excinuclease can result from either a pyrimidine dimer or a 6-4 photoproduct.

The incisions on the 3' side.

The results presented above establish that ABC excinuclease cleaves 5' to the two major DNA UV photoproducts and that, on occasion, either photoproduct can cause "atypical" incisions 5' to the damage. We wanted to characterize incisions made on the 3' side of the photoproducts causing staggered cutting on the 5' side. To this end, we labeled the 3' terminus of the same DNA strand, irradiated the DNA fragment with UV and then treated it with ABC excinuclease, photolyase plus ABC excinuclease (with and without photoreactivation), endo V or hot alkali and analyzed the reaction products on a sequencing gel. The results are shown in Figure 3. The low level of dimer formation at T₄₅T₄₆ and the relatively inefficient incision by ABC excinuclease corresponding to the dimer at this site makes the assignment of incision sites difficult; however, it appears that the 5th phosphodiester bond 3' to the photoproduct is hydrolyzed (compare lanes 10 and 11). The following observations were made regarding photoproducts at T₇₀T₇₁: ABC excinuclease hydrolyzes the 4th through 9th phosphodiester bonds 3' to this site, all but the incision at the 5th phosphodiester bond are prevented by photoreactivation. Thus, we conclude that the 6-4 photoproduct at this site causes incision of the 5th phosphodiester bond 3' to the adduct. On the other hand, pyrimidine dimer at this site apparently leads to ABC excinuclease incisions over a 6 nucleotide region. The two most distal cuts also correspond to incisions at the 3rd and 4th phosphodiester bonds 3' to the photoproduct at T₆₇C₆₈ and are, to some extent, caused by the adduct at this location. However, a comparison of the endo V and piperidine digestion patterns (lanes 9 and 10) shows that about 90% of the adducts at T₆₇C₆₈ are 6-4 photoproducts while the ABC excinuclease incision pattern of the photoreactivated DNA (lane 4) indicates that the incisions at this site are virtually eliminated by photoreactivation. We conclude that while some of the incisions at the 8th and 9th phosphodiester bonds 3' to the T₇₀T₇₁

Figure 4. The incision sites of ABC excinuclease on both sides of selected photoproducts. ABC excinuclease incision sites relative to the pyrimidine dimers ([]) and 6-4 photoproducts (2) at a) The sequence of the relevant portion of the DNA fragment used in this study; b-e, T₄₅T₄₆ (b), T₆₇C₆₈ (c), T₇₀T₇₁ (d) and T₇₄C₇₅ (e) are shown. The percentage numbers indicate the frequency for occurrence of either photoproducts at the indicated sites. The relative frequencies of the two photoproducts at each site were obtained by densitometric scanning of the *M. luteus* UV endonuclease, endo V and piperidine channels and were not corrected for position effect (Haseltine et al., 1980). Alkali hydrolysis of 5' and 3' DNA did not always yield the same value for the percentage of 6-4 photoproducts produced at a particular pair of nucleotide. These values should, therefore, only be considered to be estimates.

pyrimidine dimer are caused by the photoproduct at the neighboring T₆₇C₆₈ site, the pyrimidine dimer at this site causes the hydrolysis of the 4th, 5th, 6th, 7th, 8th or 9th phosphodiester bonds 3' to the dimer.

Figure 5. Size of the fragments removed by ABC excinuclease. Uniformly labeled ($[\alpha\text{-}^{32}\text{P}]\text{ATP}$)-UV-irradiated M13mp7 DNA was digested with ABC excinuclease and the digestion product was separated on a 20% DNA sequencing gel. Lane 1, non-irradiated DNA treated with ABC excinuclease; Lane 2, UV-irradiated DNA digested with ABC excinuclease; Lanes 3-6, Maxam-Gilbert sequence ladders (A + G, G, T + C, C) as size standards. Since the fragments generated by Maxam-Gilbert treatment contain a phosphate at both termini, they migrate approximately 1 space faster in the 10-20 nucleotide range than fragments of equal size that contain 5'-P and 3'-OH termini (the ABC excinuclease excision fragment, see reference 5).

In figure 4, we have summarized our conclusions regarding the alternate ABC excinuclease incision sites. The pyrimidine dimer at T₄₅T₄₆ causes incisions at the 6th, 7th, or 8th phosphodiester bonds 5' and the 5th phosphodiester bond 3' to the dimer. The pyrimidine dimer at T₇₀T₇₁ causes

hydrolysis of the 8th phosphodiester bond 5' and the 4th through 9th phosphodiester bonds 3' to the dimer, whereas, the 6-4 photoproduct at T₇₀T₇₁ results in incisions at either the 7th or the 8th phosphodiester bond 5' and only the 5th phosphodiester bond 3' to the photoproduct.

The size of the excised oligonucleotide.

Based on examination of incision sites in a number of terminally labeled, UV irradiated DNA fragments, it was originally proposed (5) that ABC excinuclease removes UV photoproducts in the form of 12-13 nucleotide-long oligomers. The results presented here suggest that the excinuclease may remove photoproducts by excising oligonucleotides ranging in sizes from 10 to 16 bases (e.g., incision at the 7th 5' and the 4th 3' phosphodiester bonds relative to the T₇₀T₇₁ photoproduct should produce an 11-mer). To investigate this possibility, UV irradiated DNA uniformly labeled with α -³²P dATP was digested with the enzyme and the size of the excised fragments was determined on a DNA sequencing gel. The result is shown in Figure 5. The excinuclease produces two major oligonucleotide classes which are 12 and 13 nucleotides in length. We do not, within the sensitivity of our assay, detect oligonucleotides less than 12 or greater than 13 bases in length. However, the data for Figure 5 was obtained with DNA from a different source (M13mp7) than the one used in the experiments demonstrating staggering. It proved experimentally difficult to obtain sufficient quantities of uniformly labeled (Nci I - Nru I)₁₀₂ fragment to carry out such excision experiments.

DISCUSSION

We have developed a method to study UV photoproduct distribution and frequency at specific DNA sequences. Using this method we have studied the cause of non-standard ABC excinuclease incisions in UV-irradiated DNA, the action of photolyase on various types of pyrimidine dimers and the interactions of these two enzymes at the sites of UV photoproducts. Our main conclusions are listed below.

1) ABC excinuclease generally hydrolyzes the 8th phosphodiester bond 5' and the 4th or 5th phosphodiester bond 3' to pyrimidine-pyrimidine sequences in UV irradiated DNA. However, analysis of the digestion products of terminally labeled, UV irradiated DNA fragments occasionally reveals bands on DNA sequencing gels that do not correspond to the pattern predicted from this mode of action (5). This may be due to several factors: a) incisions caused by infrequent photoproducts, b) the uncoupled hydrolyses of the two phosphodiester bonds, c) hydrolysis of phosphodiester bonds

other than the ones predicted by the standard excision mechanism. In this paper we have presented evidence for such alternate incision patterns.

2) Both pyrimidine dimers and 6-4 photoproducts, at certain locations, cause staggered incisions by ABC excinuclease. We have presented evidence that a TT dimer at a certain site is removed by hydrolysis of the 6th, 7th or 8th phosphodiester bond 5' to the dimer. Similarly, we have also shown that a 6-4 photoproduct at a certain location results in the hydrolysis by the excinuclease of the 7th or the 8th phosphodiester bond 5' to the photoproduct while a pyrimidine dimer at this location is removed almost exclusively by the hydrolysis of the 8th phosphodiester bond 5' to the dimer.

3) ABC excinuclease removes UV photoproducts in the form of 12 or 13 nucleotide-long oligomers. If the enzyme hydrolyzes, at a significant frequency, the 7th instead of the 8th phosphodiester bond on the 5' side and either the 4th or the 5th phosphodiester bond on the 3' side of UV photoproducts, one would expect to see 11-mers excised by the enzyme in addition to 12- and 13-mers. We do not detect 11-mers by our assay and, therefore, conclude that non-standard incisions are rare. However, the production of 11-mers at some low frequency probably occurs, but could not be detected with our assay which is of insufficient sensitivity.

4) ABC excinuclease efficiently removes 6-4 photoproducts from DNA. Sancar and Rupp (5) and Franklin and Haseltine (21) previously reported data obtained *in vitro* and *in vivo*, respectively, supporting the removal of 6-4 photoproducts by ABC excinuclease. In this paper we have presented data directly demonstrating that the enzyme does remove 6-4 photoproducts: the enzyme removes non-photoreactivable, alkali-labile pyrimidine-pyrimidine photoproducts.

5) *E. coli* DNA photolyase binds to pyrimidine dimers in the dark and stimulates their removal by ABC excinuclease. It was previously shown (18) that *E. coli* DNA photolyase stimulates the incision of UV-irradiated, but not of cisplatin damaged, DNA by ABC excinuclease. In this paper we have presented direct evidence that photolyase stimulates the removal of pyrimidine dimers by ABC excinuclease but has no effect on the nuclease's activity on 6-4 photoproducts. It can, therefore, be concluded that photolyase does not bind to 6-4 photoproducts.

6) Photolyase shows dimer specificity both with regard to binding and photolysis. We find that TT dimers are good substrates for both binding and photolysis and that TC, CT and in particular CC dimers are not bound

efficiently by the enzyme (lack of stimulation of ABC excinuclease activity) nor repaired efficiently (failure to remove the *M. luteus* UV endonuclease sensitive sites). The extremely slow photoreactivation of some C-containing dimers should be considered in experiments designed to discern the relative contributions of dimers and 6-4 photoproducts to UV-induced killing and mutagenesis by comparing the mutation rate and distribution before and after photoreactivation.

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