Recognition and Repair of the CC-1065-(N3-Adenine)-DNA Adduct by the UVRABC Nucleases†

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ABSTRACT: The recognition and repair of the helix-stabilizing and relatively nondistortive CC-1065-(N3-adenine)-DNA adduct by UVRABC nuclease has been investigated both in vivo with ϕX174 RFI DNA by a transfection assay and in vitro by a site-directed adduct in a 117 base pair fragment from M13mp1. CC-1065 is a potent antitumor antibiotic produced by *Streptomyces zelensis* which binds within the minor groove of DNA through N3 of adenine. In contrast to the helix-destabilizing and distortive modifications of DNA caused by ultraviolet light or *N*-acetoxy-2-(acetylamino)fluorene, CC-1065 increases the melting point of DNA and decreases the S1 nuclease activity. Using a viral DNA–*Escherichia coli* transfection system, we have found that the *uvrA*, *uvrB*, and *uvrC* genes, which code for the major excision repair proteins for UV- and NAAAF-induced DNA damage, are also involved in the repair of CC-1065–DNA adducts. In contrast, the *uvrD* gene product, which has been found to be involved in the repair of UV damage, has no effect in repairing CC-1065–DNA adducts. Purified UVRA, UVRB, and UVRC proteins must work in concert to incise the drug-modified ϕX174 RFI DNA. Using a site-directed and multiple CC-1065 modified (*Msp*I–*Bst*NI) 117 base pair fragment from M13mp1, we have found that UVRABC nuclease incises at the eighth phosphodiester bond on the 5′ side of the CC-1065–DNA adduct on the drug-modified strand. The enzymes do not cut the noncovalently modified strand. At low drug binding ratios, of the four CC-1065 binding sites identified in the (*Msp*I–*Bst*NI) 117 base pair fragment, GATTA*, GGAAA*, GATAA*, and TTTTA* (* indicates the covalently modified adenine), only the adduct at the high-affinity binding site, GATTA*, is incised by the UVRABC nucleases. No difference in the effect of CC-1065 on local DNA structure, as determined by the DNase I cleavage pattern, was evident among these sites. At high drug binding ratios, a fifth drug binding site, AGCTA*, is identified. At this concentration UVRABC nucleases are unable to incise any of these five CC-1065–DNA adducts. The DNA sequence and/or helix-stabilizing effect of multiple adducts may determine the recognition and/or incision of the drug–DNA adduct by UVRABC nuclease. These results are discussed in relation to the structure of the CC-1065–DNA adduct and the effect of drug binding on local DNA structure.

The *uvrA*, *uvrB*, and *uvrC* genes in *Escherichia coli* cells control the initial steps of excision repair of DNA damage induced by UV[1] radiation and by bulky chemical carcinogens and drugs such as NAAAF, benzo[*a*]pyrenediol epoxide, *cis*-platinum, and psoralen (Seeberg et al., 1983; Sancar et al., 1985; Beck et al., 1985). These agents induce various extents of deformation in DNA helix; DNA damaged by these agents is more sensitive to S1 nuclease digestion (Grunberger & Weinstein, 1978) and also exhibits a decreased melting temperature (Trifonov et al., 1968; Fuchs & Duane, 1974; Pulkrabek et al., 1978). Purified *uvrA*, *uvrB*, and *uvrC* gene products have been shown to work in concert in incising both the 5′ side (eighth phosphodiester bond) and the 3′ side (fifth or fourth phosphodiester bond) of the damaged base(s), such as cyclobutane dipyrimidines, ⟨6–4⟩ photoproducts, *cis*-platinum adducts, and dG-C8-AAF.

CC-1065 is a potent antitumor antibiotic produced by *Streptomyces zelensis* [Hanka et al., 1978, for recent reviews, see Hurley and Needham-VanDevanter (1986) and Reynolds et al. (1986)]. The three benzodipyrrole subunits of CC-1065 are joined by out of plane amide bridges that impart a shallow right-handed twist along the length of the molecule (Chidester et al., 1981). The overall twisted banana shape of the CC-1065 molecule presumably facilitates the nonintercalative binding of the drug in the minor groove of DNA. A presumed proximity effect between the exocylic carbon atom of the cyclopropane ring of CC-1065 and N3 of adenine in duplex DNA then leads to covalent binding (Figure 1). Although we arbitrarily represent the CC-1065 covalently modified adenine as the neutral species, it could also occur as purine species with a positive charge at one of the nitrogen atoms. As is common to N3 adenine alkylated species, thermal treatment (90 °C for 30 min) at neutral pH leads to cleavage of the N-glycosidic linkage and subsequent strand breakage at the AP site (Reynolds et al., 1985) (Figure 1). This strand breakage assay has been used to determine the sequence specificity of CC-1065 (Reynolds et al., 1985). In contrast to "UV-like" agents as described above, CC-1065 *increases*

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[1] Abbreviations: UV, ultraviolet light; NAAAF, *N*-acetoxy-2-(acetylamino)fluorene; dG-C8-AAF, *N*-(deoxyguanos-8-yl)-2-(acetylamino)fluorene; bp, base pair; *N*-OH-AF, *N*-hydroxy-2-aminofluorene; BAP, bacterial alkaline phosphatase; PNK, polynucleotide kinase; AP, apurinic acid; NOESY, nuclear Overhauser enhancer spectroscopy; MPE, methidiumpropyl-EDTA; TAE, 50 mM Tris, 5 mM sodium acetate, and 1 mM EDTA, pH 7.6; dTTP, thymidine triphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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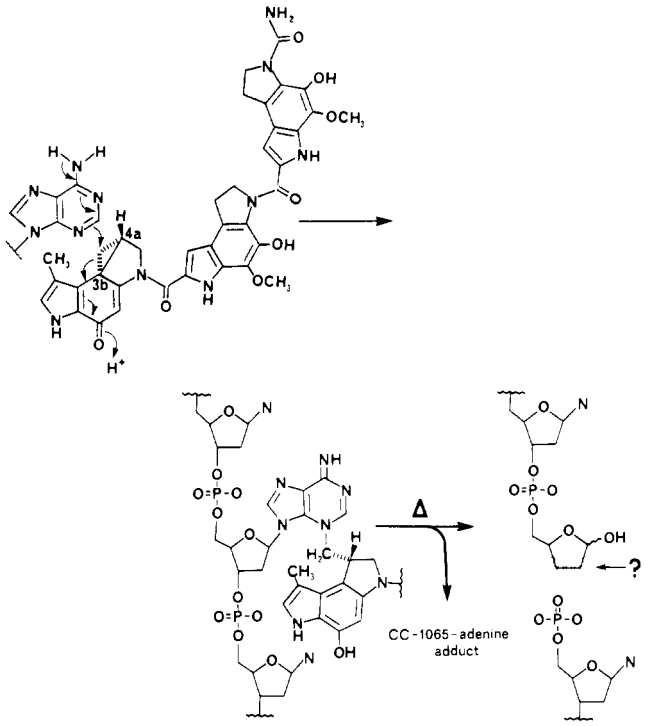

FIGURE 1: Reaction of CC-1065 with N3 of adenine in DNA and products from the thermal cleavage reaction. The exact nature of the species generated on the 5′ side of the strand break is unknown.

the melting point of DNA and *decreases* the sensitivity to S1 nuclease (Swenson et al., 1982). The stabilization of the helix to S1 nuclease and thermal denaturation is consistent with molecular models of the CC-1065-(N3-adenine)-DNA adduct (Figure 2) that show a snug fit of the drug in the minor groove spanning a four base pair region to the 5′ side of the covalently modified adenine (Hurley et al., 1984). Recently, we have prepared a site-directed CC-1065–DNA adduct in which CC-1065 is bound to A-6229 in a 117-bp restriction fragment from M13mp1 (Needham-VanDevanter & Hurley, 1986). DNase I and restriction enzyme analysis of this single adduct in the 117-bp fragment shows that CC-1065 produces an asymmetric effect on DNA structure such that the conformation of the covalently modified strand of CC-1065 is proposed to be altered up to about one helix turn to the 5′ side of A-6229 (Hurley et al., 1987).

The unique features of the CC-1065-(N3-adenine)-DNA adduct have prompted us to investigate whether this well-defined lesion may be useful in elucidating the structural and electronic features responsible for recognition of covalently modified DNA by UVRABC nucleases. The results show that while all three proteins are required for both in vivo and in vitro recognition and repair, the efficiency of recognition in vitro is poor in contrast to that of more distinctive DNA adducts. These results are discussed in relation to the known effects of CC-1065 on local DNA structure.

Materials and Methods

DNA Transfection. E. coli strains MST13 (*uvrA6*), MST3 (*uvrB5*), MST8 (*uvrC34*), and MST17 (*uvrD3*) were constructed by P1 or T4GT7 transduction (Tang et al., 1982). The parental strain HF4704 (MST1 wild type) was obtained from Dr. T. Kunkel. The purification of ϕX174am3cs70 RFI DNA and the DNA transfection were performed exactly as described previously (Tang et al., 1982).

Purification of UVRA, UVRB, and UVRC Proteins. UVRA, UVRB, and UVRC proteins were purified from *E. coli* K12 strain CH296 (*recA endA1*/F′*lacI*^{Q}) containing UVR protein overproducing plasmids pUNC45 (*urvA*), pUNC211 (*urvB*), and pDR3274 (*uvrC*). These plasmids and the *E. coli* strains were gifts of Dr. A. Sancar. The details of the purification procedure will be published elsewhere. UVRD proteins were gifts of Dr. T. Lohman. All the proteins were purified to the extent shown in Figure 3.

^{32}*P 5′ or 3′ End Labeling.* (*a*) *Site-Directed CC-1065–DNA Adduct.* The labeling method was performed as described previously (Needham-VanDevanter & Hurley, 1986).

(*b*) *Multiple CC-1065–DNA Adduct.* The 250-bp fragments isolated from *Msp*I digestion of M13mp1 were 5′ ^{32}P end labeled by sequential BAP and T4 PNK treatment. DNA fragments in 50 μL of 50 mM NaCl and 50 mM Tris-HCl, pH 8.0, were incubated with 1 unit of BAP for 1.5 h at 65 °C followed by phenol/chloroform extraction and ethanol precipitation. The DNA pellets were resuspended in 25 μL of 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM spermidine, and 0.5 mCi of [γ-^{32}P]ATP to

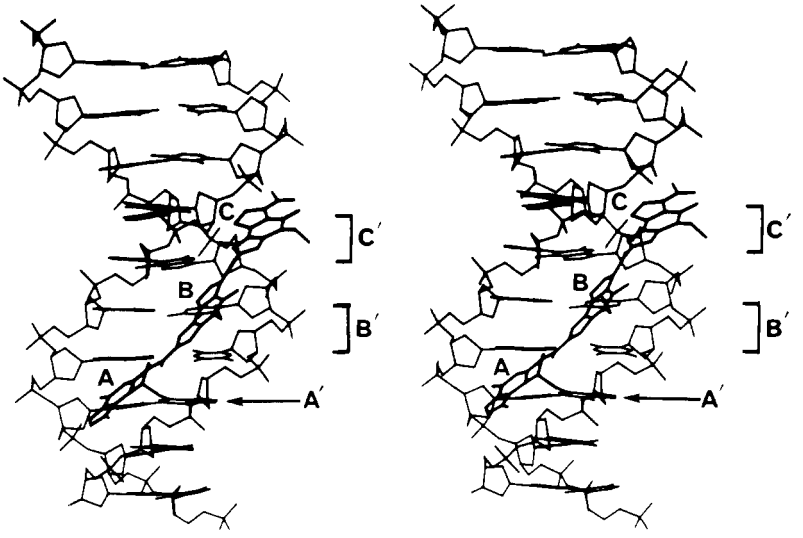

FIGURE 2: Stereo diagram of a CC-1065–DNA adduct. The computer graphic representation of a CC-1065–DNA adduct using the X-ray crystal coordinates for ring-opened CC-1065 (Chidester et al., 1981) and B-form DNA generated by the MIDAS graphics program. The sequence used is that of the center of a 14 base pair oligo deoxy duplex shown to bind a single CC-1065 molecule. σ bonds around amide linkages between the drug subunits (see Figure 1) were rotated to reduce close drug–DNA contacts upon docking. CC-1065 A, B, and C subunits are labeled.

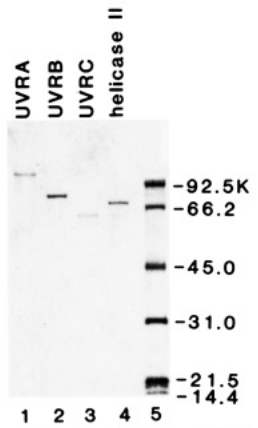

FIGURE 3: Gel electrophoresis of purified UVRA, UVRB, UVRC, and UVRD (helicase II) proteins. The proteins were electrophoresed in a 10% polyacrylamide gel with sodium dodecyl sulfate and were stained with Coomassie blue.

which 4 units of T4 PNK was added. Samples were incubated 20 min at 37 °C and precipitated by ethanol. A second digestion by *Bst*NI was carried out to obtain the 117-bp fragment. For 3′ end labeling, the 137-bp fragments obtained from *Bst*NI digestion of M13mp1 RF DNA were dissolved in 30 μL of 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 7.5 mM dithiothreitol, and 0.1 mCi of [α-^{32}P]dTTP. One unit of DNA polymerase I (Klenow fragment) was added and incubated for 20 min at room temperature. After ethanol precipitation, a secondary digestion by *Msp*I was carried out to obtain the 117-bp fragments.

Modification of DNA with CC-1065. (*a*) *φX174 RFI DNA.* Purified φX174 RFI DNA was reacted with various concentrations of CC-1065 in DSC solution (15 mM NaCl, 1.5 mM sodium citrate, pH 7.4) at room temperature for 90 min to produce various extents of modification.

(*b*) *Site-Directed CC-1065–DNA Adduct.* Construction of the site-directed CC-1065–DNA adduct was performed as described previously (Needham-VanDevanter & Hurley, 1986).

(*c*) *Multiple CC-1065–DNA Adduct.* Various 10-fold dilutions of a standard 280 μM solution of CC-1065 was added to 5′ or 3′ ^{32}P-labeled (*Msp*I–*Bst*NI) 117-bp fragment of M13mp1 and incubated at 37 °C for 2 h.

All the above modifications of DNA by CC-1065 were terminated by ethanol precipitation.

MPE–Fe(II) or DNase I Footprinting and Thermal Breakage of CC-1065-Bound Fragments. The (*Msp*I–*Bst*NI) 117-bp fragments 5′ ^{32}P end labeled on the (+) strand were digested with MPE–Fe(II) (Needham-VanDevanter & Hurley, 1986), subjected to thermal treatment (90 °C, 30 min) (Reynolds et al., 1985), or footprinted with DNase I (Hurley et al., 1987) to confirm both the location and the orientation of the CC-1065–DNA adduct according to the method described previously (Needham-VanDevanter & Hurley, 1986).

Reaction of UVR Protein with CC-1065-Modified DNA. For the incision assay, UVR proteins (0.2 pmol of UVRA, 2.2 pmol of UVRB, and 0.28 pmol of UVRC) were added singly or in combination to CC-1065-modified φX174 RFI DNA (0.2 μg) in a reaction mixture which contained 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 85 mM KCl, 1 mM ATP, and 1 mM dithiothreitol. The incubations were conducted at 37 °C in the dark for 90 min. We have found that under these conditions UVRABC nucleases incise the drug-modified φX174 RFI DNA optimally. The reactions were stopped by the addition of sodium dodecyl sulfate (0.1%) and heated at 65 °C for 2 min. The resultant DNAs were subjected to electrophoresis in 1% agarose in TAE buffer at 1 V/cm for 16 h. For the ^{32}P-labeled DNA fragment, the amount of UVR proteins added to the reaction mixture and the incubation conditions were the same as described above, except that the reactions were stopped by phenol/chloroform extractions and subsequently ethanol precipitation. The UVR protein reacted DNA fragments were denatured by heat (90 °C, 2 min) in formamide (80%)–NaOH (10 mM) solution and then subjected to sequencing electrophoresis in parallel with Maxam and Gilbert (1980) A+G and T+C reactions.

RESULTS

uvrA, *uvrB*, and *uvrC* genes control the excision repair of UV-induced and bulky chemical carcinogen induced DNA damage in both chromosomal and extrachromosomal DNA (Hanawalt et al., 1979). This conclusion is mainly derived from the following results: (1) compared to wild-type cells, *uvrA*, *uvrB*, and *uvrC* mutant cells are sensitive to cell death when exposed to these DNA damaging agents (Howard-Flanders et al., 1964), and (2) UV-irradiated or chemical carcinogen modified phage, phage DNA, or plasmid DNA has a lower infectivity, transfectivity, or transformation ability in *uvrA*, *uvrB*, or *uvrC* mutant cells in comparison with wild-type cells (Tang et al., 1982; Fuchs & Seeberg, 1983; Taketo et al., 1979).

Transfection Experiments with CC-1065-Modified φX174 RFI DNA in E. coli uvr Mutant Cells. In order to test the role of *uvrA*, *uvrB*, and *uvrC* genes in the repair of CC-1065-induced DNA damage, purified *φX174* RFI DNA was reacted with different concentrations of CC-1065 for transfection assay. The number of CC-1065 adducts per molecule of DNA could not be calculated because radioactively labeled CC-1065 is not available. However, we have found that under the reaction conditions we have used the number of DNA adducts formed is proportional to the concentration of CC-1065 (unpublished results). When these CC-1065-modified DNAs were transfected into *E. coli*, the transfectivity was inversely proportional to the concentration of CC-1065 used for modification (Figure 4). These modified DNAs have a lower transfectivity in *uvrA*, *uvrB*, and *uvrC* mutant cells compared with wild-type cells. However, the transfectivities of the modified DNA in *uvrD* mutant cells are the same as in wild-type cells. These results indicate the *uvrA*, *uvrB*, and *uvrC*, but not *uvrD*, gene products are involved in the repair of CC-1065-induced DNA damage.

In Vitro Repair of CC-1065–DNA Adducts by UVRABC Nucleases in φX174 RFI DNA. In order to test whether these gene products work in coordination or independently in repairing the CC-1065–DNA adducts, purified UVRA, UVRB, and UVRC proteins singly or in combination were reacted with CC-1065-modified φX174 RFI DNA in the presence of ATP and Mg^{2+} ion. Any repair process that causes a DNA strand breakage will convert the supercoiled RFI DNA to relaxed RFII or linear DNA. The electrophoretic mobilities of these three kinds of DNA are significantly different (Figure 5). The results in Figure 5 show that these three proteins must work together to cut the CC-1065-modified DNA (compare lane 8 to lanes 1–7). The background cuttings induced by adding UVR proteins singly or in pairs are likely due to nonspecific endonuclease contamination in the UVR protein preparations, since UVR proteins, singly or in pairwise combination, do not induce DNA strand breaks corresponding to CC-1065 modification sites in DNA sequencing gels (data not shown). The addition of UVRD protein over a concentration range from 1.3×10^{-3} to 2.5×10^{-2} pmol has no effect on the activity of UVRABC nucleases toward CC-1065-modified DNA (Figure

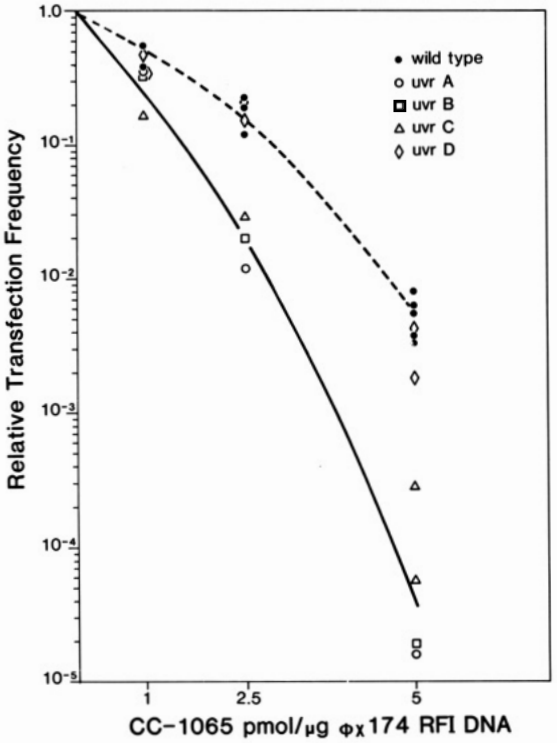

FIGURE 4: Relative transfectivity of CC-1065-modified ϕX174 RFI DNA in wild-type *E. coli* (●) and *uvrA* (○), *uvrB* (□), *uvrC* (△), and *uvrD* (◇) mutant cells.

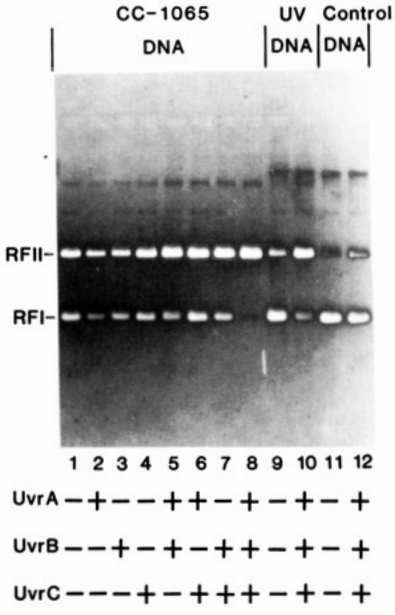

FIGURE 5: Incision of CC-1065-modified ϕX174 RFI DNA by UVRABC nucleases. UVRA, UVRB, and UVRC protein was added singly or in combination as indicated by (+) and (–) signs to CC-1065-modified ϕX174 RFI DNA (lanes 1–8) solution. The reaction conditions were described in the text. Lanes 9 and 10 represent UV-irradiated ϕX174 RFI DNA, and lanes 11 and 12 represent control ϕX174 RFI DNA.

6). We have found that the amount of UVRD protein used in this experiment enhances UVRABC nuclease activity toward UV- and *N*-OH-AF-induced DNA damage by 50 and 200%, respectively (Tang et al., unpublished results).

Recognition and Incision of Site-Directed CC-1065-(N3-Adenine)-DNA at A-6229 in a 117-bp Fragment by the UVRABC Nucleases. It has been found that UVRABC nucleases incise both at the 5′ side (eighth phosphodiester bond) and at the 3′ side (fifth or fourth phosphodiester bond) of

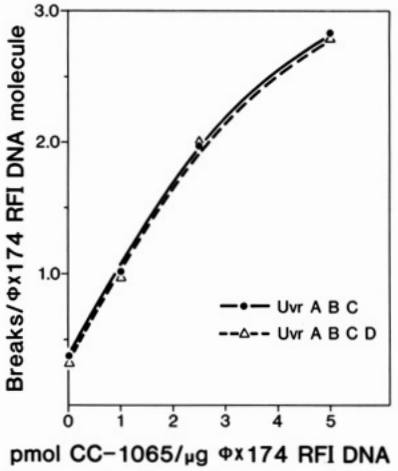

FIGURE 6: UVRD protein effect on the incision of CC-1065-modified ϕX174 RFI DNA by UVRABC nucleases. CC-1065-modified ϕX174 RFI DNAs were reacted with UVRABC nucleases in the presence (dotted line) or absence (solid line) of UVRD protein (2.5×10^{-3} pmol). The amount of UVR proteins and DNA used and the incubation conditions are the same as in Figure 5.

cyclobutane dipyrimidine, ⟨6–4⟩ photoproduct, psoralen adduct, and dG-C8-AAF (Sancar & Rupp, 1983; Yeung et al., 1983; Sancar et al., 1985). It is of interest to determine whether UVRABC nucleases have the same mode of action toward the CC-1065-induced DNA damage, which causes greatly different effects on the DNA structure from UV- and NAAAF-induced DNA damage. The site-directed CC-1065-(N3-adenine)-DNA adduct of A-6229 in the 117-bp fragment from M13mp1 was prepared either 3′ or 5′ labeled on either the covalently (+) or noncovalently (–) modified strands (Needham-VanDevanter & Hurley, 1986). To demonstrate the presence of a single CC-1065–DNA adduct at A-6229, thermal treatment (90 °C for 30 min) or MPE–Fe(II) footprinting analysis was carried out as described previously (Needham-VanDevanter & Hurley, 1986). The results of this analysis for the (+) strand are shown in Figure 7. Lane 5 shows the single-strand breakage site at A-6229 and lane 3 the MPE–Fe(II) footprinting to the 5′ side of A-6229. A similar experiment with the (–) strand does not reveal any additional strand breakage sites, but a MPE–Fe(II) footprint opposite the expected site-directed adduct site at A-6229 is found (Needham-VanDevanter & Hurley, 1986).

When the same or similarly constructed site-directed CC-1065–DNA adducts were reacted with the UVRABC nucleases, the results examplified by those in Figure 8 were obtained. The UVRABC nucleases did not cut the (–) strand (lane 3) while enzyme-mediated incision was obtained in both CC-1065-modified 5′ and 3′ (+) strands (lanes 6 and 9, respectively). We, therefore, conclude that the UVRABC nucleases are able to differentiate between the covalently and noncovalently modified strands. For both the 5′ and 3′ labeled fragment modified at A-6229 with CC-1065 and subjected to the UVRABC nucleases, two strand breakage sites are evident. In each case one of these is due to the thermally induced strand breakage at A-6229 while the other is a 5′ incision by the UVRABC nuclease. The absence of a UVRABC-induced incision on the 3′ side of the CC-1065 adduct in the 3′ labeled fragment (lane 9), together with the 5′ incision site at seven nucleotides to the 5′ side of A-6229, proves that the UVRABC nuclease is only able to cut on one side (5′) of the CC-1065–DNA adduct. This is in stark contrast to the mode of action of UVRABC nucleases on other drug– or carcinogen–DNA adducts (see before). Moreover, the majority of the radioactive material remains at the high end of the gel, demonstrating that

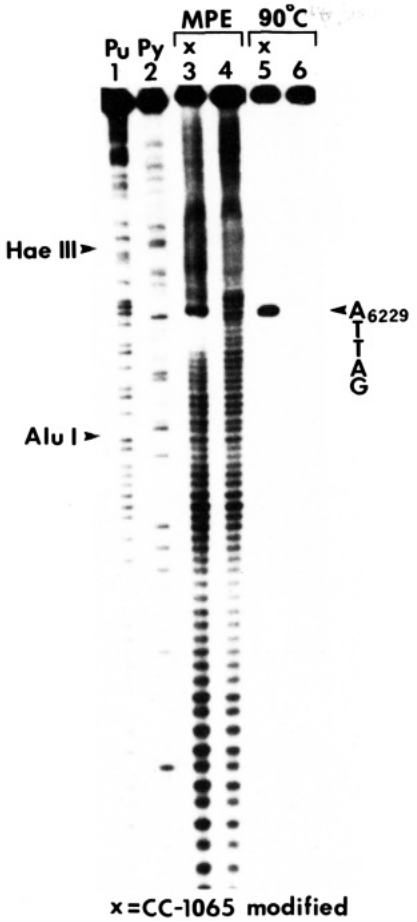

FIGURE 7: Thermal strand break and MPE–Fe(II) footprinting analysis of the site-directed CC-1065–DNA adduct. Single 5′ ^{32}P-labeled site-directed CC-1065–DNA adducts were analyzed for CC-1065 adduct location by the thermal strand scission assay (lanes 5 and 6) and MPE–Fe(II) digestion (lanes 3 and 4). Analyses were performed on 5′ ^{32}P-labeled (+) strand. DNA fragments with (lanes marked X) or without CC-1065 modification. Reactions were electrophoresed adjacent to unmodified DNA (lanes 4 and 6) and Maxam–Gilbert sequencing reactions (lanes 1 and 2).

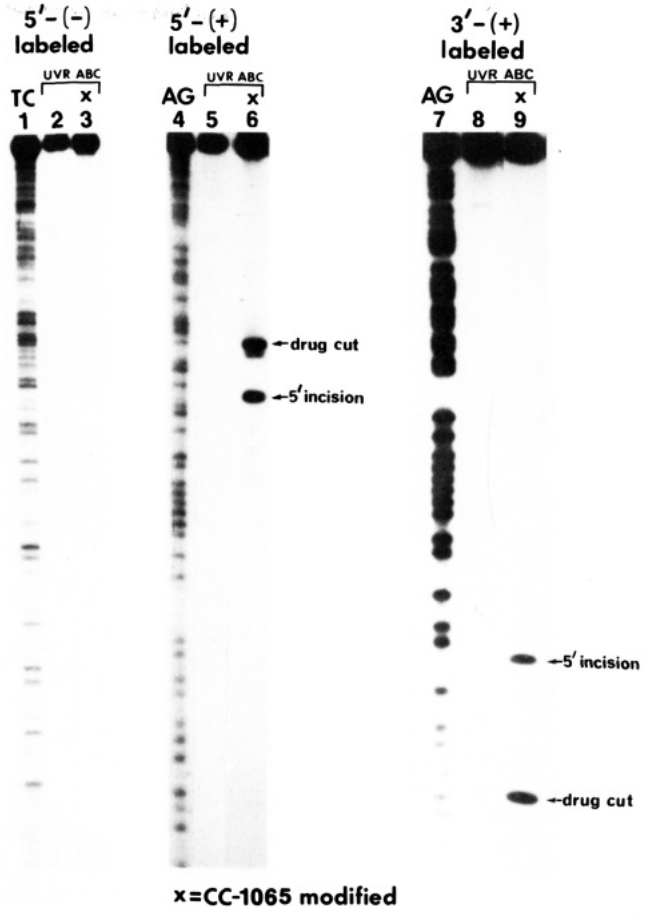

FIGURE 8: Reaction of UVRABC nucleases with 5′ and 3′ labeled (*Msp*I–*Bst*NI) 117-bp fragment with site-directed CC-1065–DNA adducts. (Lanes 1, 4, and 7) Maxam–Gilbert sequencing reactions. (Lane 2) Unmodified 5′ labeled (−) DNA fragment. (Lane 3) 5′ labeled site-directed adduct fragment. (Lane 5) Unmodified 5′ labeled (+) fragment. (Lane 6) 5′ labeled site-directed adduct (+) fragment. (Lane 9) 3′ labeled site-directed adduct (+) fragment. (Lane 8) Unmodified 3′ labeled (+) DNA fragment. (Lanes 2, 3, 5, 6, 8, and 9) DNA reacted with UVRABC nucleases.

UVRABC cleavage at CC-1065 binding sites is not very efficient.

Recognition and Incision of Multiple CC-1065–DNA Adducts by the UVRABC Nucleases in the 117-bp Fragment. In order to determine whether CC-1065-(N3-adenine)-DNA adducts in different sequences were equally well recognized by the UVRABC nucleases, the same restriction enzyme fragment was modified with CC-1065, without the site-directed adduct strategy. We have previously shown for this fragment that the high affinity binding sites are all found on the (+) strand (Needham-VanDevanter & Hurley, 1986). The same four CC-1065 binding sites are evident in both 3′ and 5′ labeled fragments (Figure 9). When these CC-1065-modified fragments were incubated with UVRABC nucleases (the results are shown in Figure 9, lanes 5 and 12), only one additional strand cleavage site is evident over the CC-1065 thermal cleavage sites present in lanes 4 and 11. This cleavage site corresponds to the same nuclease incision site seen in Figure 8 for the site-directed adduct; i.e., UVRABC incision occurs exclusively to the 5′ side of the CC-1065 covalent adduct site at a distance of seven nucleotides from A-6229. There are at least two possibilities to account for the absence of UVRABC nuclease incision at CC-1065 binding sites (GATAA*, GGAAA*, and TTTTA*) other than the A-6229 position: (1) CC-1065 does not induce equivalent effects on local DNA structure at the various binding sites, and (2) the multiple CC-1065 adducts on either the (+) or (−) strand inhibit UVRABC cleavage. To examine whether CC-1065 might produce differential effects on local DNA structure that could produce the apparent adduct specificity for cleavage by the UVRABC nuclease, we have used DNase I as a probe of drug-induced conformational changes of DNA. Fortuitously, all of the high-affinity covalent binding sites for CC-1065 occur on the same strand, and consequently, the DNase I cleavage pattern on the (−) or noncovalently modified strand is not complicated by the presence of residual CC-1065–DNA adducts as it would be on the (+) strand. Using the site-directed adduct, we have previously demonstrated that the DNase I inhibition/enhancement pattern on the (−) strand is a reflection of events occurring on the (+) strand (Hurley et al., 1987). The results of the DNase I footprinting are shown in Figure 10. The same pattern of cleavage inhibition over a 12–15-base region and enhancements at the 3′ terminus of the inhibition zone are seen for all four binding sites. Therefore, if the selectivity of UVRABC nuclease cleavage for the CC-1065 at A-6229 is due to differences in effect of CC-1065 on local DNA structure, it escapes detection by the DNase I footprinting method.

The second possibility is that the presence of more than one CC-1065–DNA adduct inhibits recognition and/or incision by the UVRABC nucleases. Consistent with this notion are the results shown in Figure 9, which show that the adduct at

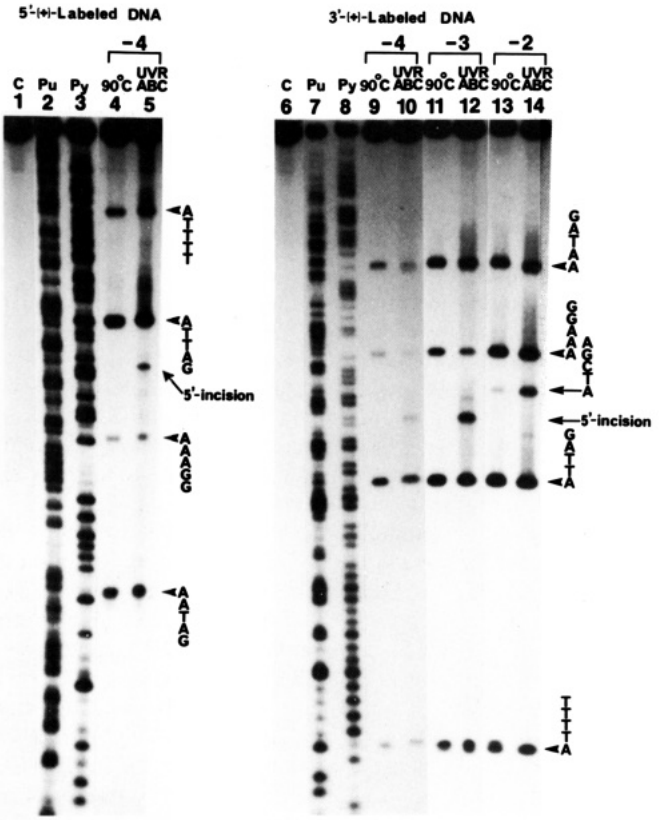

FIGURE 9: Reaction of UVRABC nucleases with 5′ (left panel) or 3′ (right panel) end labeled (*Msp*I–*Bst*NI) 117-bp fragment with multiple CC-1065 adducts. Lanes 2 and 7 and lanes 3 and 8 are Maxam and Gilbert sequencing reactions for purines and pyrimidines, respectively. Lanes 4, 5, 9, and 10 are treated with 0.028 μM CC-1065, lanes 11 and 12 are treated with 0.28 μM CC-1065, and lanes 13 and 14 are treated with 2.8 μM CC-1065. (Lanes 4, 9, 11, and 13) Modified fragments with thermal treatment (90 °C, 30 min) in DSC. (Lanes 5, 10, 12, and 14) Modified fragments with UVRABC nucleases and subsequent thermal treatment (90 °C, 2 min) in formamide–alkaline dye as described in the text. (Lanes 1 and 6) Unmodified fragments with the same UVRABC nucleases and thermal treatment as in lanes 5, 10, 12, and 14. The precise CC-1065 binding and UVRABC incision sites are indicated by arrows.

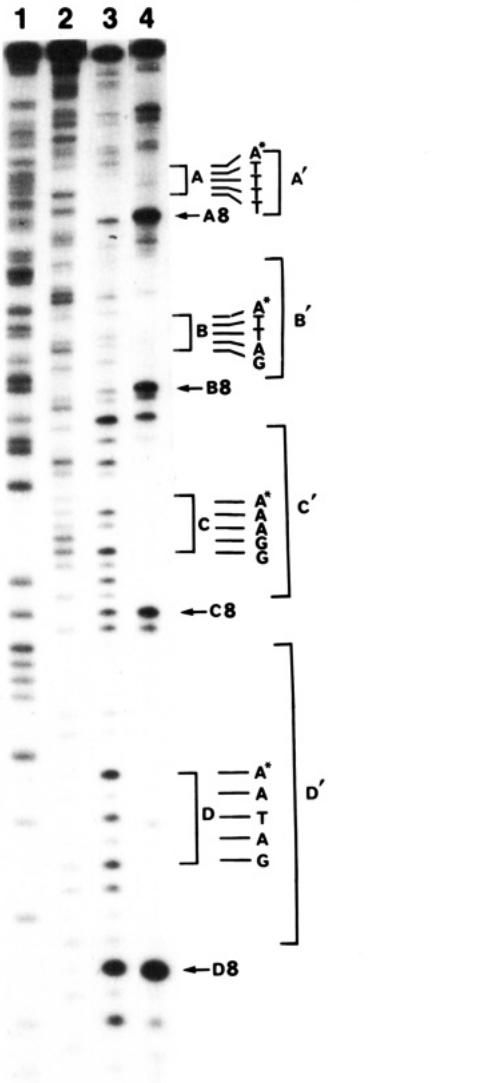

FIGURE 10: DNase I footprinting of 3′ ^{32}P-labeled (–) strand of the (*Msp*I–*Bst*NI) 117-bp fragment with multiple CC-1065–DNA adducts from M13mp1 DNA. Lanes 1 and 2 are Maxam and Gilbert purine- and pyrmidine-specific cleavage. Lanes 3 and 4 have been subjected to DNase I digestion. Lane 3 is the control (no drug modification); lane 4 has CC-1065 modification at sites indicated by brackets A, B, C, and D. These brackets correspond to the CC-1065 binding sequences that are identified as they correpond to the covalently modified (+) strand, with the covalently modified adenine denoted by an asterisk. DNase I enhanced cleavage sites are indicated by arrows, and A8 etc. refer to the corresponding CC-1065 binding site and distance between the covalent binding site and the enhancement site. The vertical bars represent the zones of DNase I inhibition corresponding to each drug binding site, i.e., A′ corresponding to CC-1065 binding site A.

A-6229 is recognized in the DNA fragments modified with 280×10^{-4} to 280×10^{-3} μM but not 280×10^{-2} μM CC-1065. A fifth drug binding site appears at position A-6217, in DNA modified with 280×10^{-2} μM CC-1065. This extra drug binding site may interfere with the recognition and/or incision of adduct at A-6229 by UVRABC nucleases.

There are weak binding sites at complementary sequences of GATAA and TTTTA (but not GGAAA); however, since the CC-1065 adduct cradles in the minor groove, it is expected that the drug binding at GATAA, and TTTTA, physically excludes the possibility of binding at their complementary sequence by another drug. The fragments with drug-induced breakage at GATAA*, GGAAA*, and TTTTA* sites (Figure 9) likely represent the populations of DNA fragments with CC-1065 binding at these sites but not at their complementary sequences. Therefore, it is unlikely that the inability of UVRABC to incise at GATAA and TTTTA sites results from the drug binding at the opposite strand, which would consequently interfere with the incision.

Discussion

The versatility of the *uvr* excision repair system in *E. coli* cells in repair of DNA damage has long been recognized; however, the rules that determine the recognition of DNA damage by the UVRABC nuclease complexes are still largely unknown. The purpose of our current study is to characterize some of the factors that influence DNA damage recognition by using purified repair proteins and a DNA construct with a site-directed adduct of antitumor antibiotic CC-1065. The effects of CC-1065 on local DNA structure have been partially characterized recently in one of our laboratories (Hurley et al., 1987). We describe the result of in vivo experiments that establish the importance of the UVRABC nucleases in repair of CC-1065–DNA adducts and in vitro experiments that characterize the recognition and incision pattern using a site-directed CC-1065–DNA adduct. The complications of using a multiple CC-1065–DNA adduct are also discussed. The relationship of these results to the established structure of the covalent CC-1065–DNA adduct and effect of drug binding on local DNA structure is explored. Interpretation

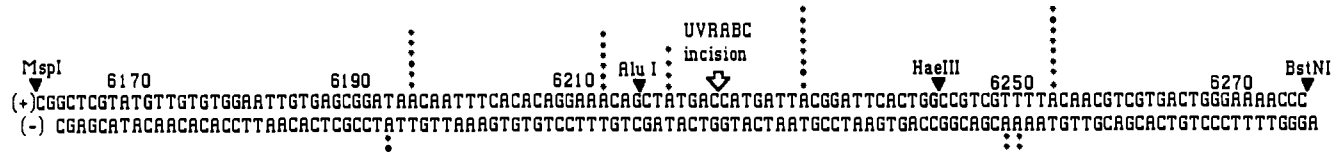

FIGURE 11: DNA sequence of (*Msp*I–*Bst*NI) 117-bp fragment. The CC-1065 binding sites are indicated by (*), and the number of asterisks represents the relative affinity of drug binding. The UVRABC incision site is indicated by an arrow. *Alu*I and *Hae*III restriction enzyme sites are indicated by (▼). The numerical system is the same as in M13mpI.

of these results provides important information on recognition of DNA damage by UVRABC nucleases.

The results of the transfection experiments with CC-1065-modified ϕX174 RFI DNA in *uvrA*, *uvrB*, and *uvrC* mutant cells compared to wild type establish the importance of the UVRABC nuclease system in recognition and repair of CC-1065-damaged DNA. Although we were not able to accurately quantify the CC-1065 lesions on ϕX174 RFI DNA, it appears that, in comparison to NAAAF-modified or UV-irradiated DNA (Tang et al., 1982), the UVRABC systems may not be so critical for phage survival, suggesting that there may be other, as yet unidentified, repair systems involved in repair of CC-1065-modified DNA. Consistent with this notion are the results from in vitro experiments using both the site-directed adduct and multiple adducts of CC-1065 in the 117-bp fragment which demonstrate that the incision efficiency is relatively low in comparison to those of other DNA adducts such as dG-C8-AAF or cyclobutane dipyrimidines. In various experiments the efficiency of UVRABC incision at the site-directed CC-1065–DNA varied between about 5 and 20%. Interestingly, Jacobson et al. (1986) recently found that *Xeroderma pigmentosum* cells, which are deficient in repair of UV-induced and polycyclic carbon induced DNA damage (Hanawalt et al., 1987), show normal nicotinamide adenine dinucleotide depletion as normal human cells after CC-1065 treatment. These results indicate that in human cells there are different repair pathways for repairing UV- and CC-1065-induced DNA damage. A second important difference from damage caused by UV (Kumura et al., 1985; Husain et al., 1985; Caron et al., 1985) and *N*-OH-AF (Tang et al., unpublished results) is the lack of enhanced UVRABC activity both in vivo and in vitro toward CC-1065-damaged DNA in the presence of the UVRD protein. Thus, while both in vivo and in vitro experiments demonstrate that all three proteins of the UVRABC nuclease are required for recognition and repair of CC-1065–DNA adducts, there are important differences from other DNA damage examined in this system.

By virtue of the availability of a single CC-1065 site-directed adduct in a 117-bp fragment, we were able to unambiguously describe the UVRABC nuclease cutting pattern in the vicinity of a CC-1065–DNA lesion. The results, which are summarized in Figure 11, reveal a single incision at seven nucleotides to the 5′ side of the covalently modification is produced by the UVRABC nucleases. While the selectivity toward the covalently modified strand was anticipated, the single incision on the 5′ side rather than breaks to both the 3′ and 5′ sides was unexpected. In other cases, such as cyclobutane dipyrimidines, ⟨6–4⟩ photoproducts, psoralen, *cis*-platinum–DNA adducts, and dG-C8-AAF lesions, cuts are found on both sides of the adducts (Sancar & Rupp, 1983; Sancar et al., 1985; Yeung et al., 1983). We have excluded the trivial explanation that the UVRABC nucleases we have prepared cut only the 5′ side of the damaged base(s) since we have found that these proteins cut both the 5′ and 3′ side of dG-C8-AAF (Tang and Pierce, unpublished results). This difference is unlikely to be due to the effect of physical hindrance by drug binding in the minor groove, since our MPE–Fe(II) footprinting results have demonstrated that bound CC-1065 points toward the 5′ end from the covalent binding site rather than toward the 3′ end. Last, and again unexpectedly, we show that there is a wide discrepancy of efficiency of UVRABC nuclease incision among the adducts formed on DNA located at different sequences. Using DNase I as a probe to monitor the effect of CC-1065 on local DNA structure for each of the four different CC-1065–DNA lesions, we do not detect any significant differences.

Identification of the factors that determine the recognition of DNA adducts by the UVRABC nuclease is dependent upon an accurate and complete picture of the ligand-modified base as well as determination of the effect of DNA binding on local DNA structure and possibly dynamics. Other factors such as superhelical density and whether the lesion is in a region of DNA available for expression are probably also important considerations for repair of drug-modified cellular DNA. While we do not have a complete picture of the in vitro CC-1065–DNA adduct, the covalent structure of adducts is known (Hurley et al., 1984), and a gross appreciation for the effect of CC-1065 binding on local DNA structure is known from studies using nuclease cleavage probes of the site-directed adduct at A-6229 (Hurley et al., 1987).

CC-1065 binds snugly in the minor groove of DNA, covalently attached through N3 of adenine (Figures 1 and 2). In contrast to other minor groove binding agents such as anthramycin (Hurley et al., 1981), which binds through the exocyclic amino group of guanine and consequently does not disturb the electronic character of the purine base, CC-1065 binding at N3 of adenine is expected to have a profound effect on the electronic character of adenine. The CC-1065–DNA adduct may be represented in the imino tautomeric form with N3 quanternized or as the neutral amino tautomeric form as shown in Figure 1. As a consequence of covalent binding to the "A" subunit of CC-1065, it is perhaps not surprising that the local DNA structure in the vicinity of the adduct is changed dramatically. Two-dimensional ^{1}H NMR NOESY experiments confirm that the inside edge of CC-1065 lies in the vicinity of the floor of the minor groove of DNA to the 5′ side of the covalent binding site (Scahill et al., 1986). However, using DNase I and restriction enzyme cleavage analysis of the same site-directed CC-1065–DNA adduct investigated in this study, we have shown that drug binding has an asymmetric effect on local DNA structure. While changes in the DNase I cleavage pattern reveal some changes in local structure on the noncovalently modified strand, the major change appears to occur on the covalently modified strand to the 5′ side of the adduct site. An *Alu*I restriction enzyme cleavage site between G-6214 and C-6215 (15 bp to the 5′ side of the covalently modified adenine) is inhibited from cleavage in the presence of CC-1065 but just on one (–) strand of DNA. The DNase I inhibition pattern shows a footprint over a 12 base pair region on the same strand. On the basis of the X-ray structure of DNase I (Suck & Oefner, 1986) and the structure of type II restriction enzyme typified by *Eco*RI (McClarin et al., 1986), we interpret these results to mean that the binding of CC-1065 to DNA changes the structure of the covalently

modified strand to the 5′ side of the adduct over about one and one-half turns of DNA, (Hurley et al., 1987). Previously we have shown by inhibition of S1 nuclease activity and an increase in thermal melting temperature of CC-1065–DNA adducts (a single adduct in 27-bp duplex increases the ΔT_m by 15–20 °C) (Needham-VanDevanter & Hurley, 1986; Swenson et al., 1982) that CC-1065 dramatically stabilizes the helical structure of DNA.

On the basis of the UVRABC nuclease incision sensitivity and cleavage pattern of the site-directed CC-1065–DNA adduct and our knowledge of the CC-1065–DNA adduct structure, it is possible to draw some important conclusions about the rules for recognition of DNA damage by the UVRABC nuclease system. First, the presently accepted recognition spectrum for the UVRABC nuclease should be extended to include the relatively nondistortive and helix-stabilizing type of adduct examplified by CC-1065. We note, also, that other covalent minor groove stabilizing and nondistortive lesions such as the anthramycin-(N2-guanine)-DNA adduct are also recognized by the UVRABC nuclease system (Tang et al., unpublished results). Furthermore, the strand selectivity of UVRABC nuclease activity for CC-1065–DNA adducts may be related to the electronically altered base on the (+) strand.

The inability of the UVRD gene product to enhance the UVRABC nuclease activity is most probably related to the absence of or even CC-1065-mediated reduction in the ability to form single-stranded DNA in the vicinity of the CC-1065–DNA adduct. This may be a common feature of helix-stabilizing adducts such as CC-1065 and anthramycin, which likewise shows no UVRD enhancement of UVRABC nuclease activity (Tang et al., unpublished results).

The relatively low efficiency of UVRABC nuclease cleavage of CC-1065–DNA adducts in contrast to other covalent adducts may be due to the stabilizing effect of the adduct and/or to the relatively nondistortive effect of CC-1065 on local DNA structure. This may also be related to the inability to cleave on the 3′ side of CC-1065–DNA adduct. Significantly, there is a correlation between DNA distortion as measured by inhibition of DNase I and *Alu*I cleavage and cleavage by the UVRABC nuclease on the 5′ side of the adduct.

Why the UVRABC nuclease recognizes the CC-1065–DNA adduct at A-6229 but fails to recognize other CC-1065–DNA adducts on the same fragment is an important question. Since the covalently modified adenines in -TTTTA*- and -GATAA*- are 23 and 33 bases, respectively, away from the ends of the fragment, it is unlikely that the inability of UVRABC nucleases to cut CC-1065–adenine adducts in these sequences is due to the proximity of the adducts to the end of the DNA fragment since Van Houten et al. (1986) have shown that UVRABC nucleases are able to cut DNA damage 14 bp from the DNA terminal. In some experiments a minor amount of cleavage on the 5′ side was noted with the other CC-1065–DNA adducts, but the adduct at A-6229 was always recognized and cleaved at the 5′ side well in excess of cleavage of the other CC-1065–DNA adducts, except at drug concentrations in which a fifth CC-1065 binding site appears in DNA. The DNase I cleavage patterns of the various CC-1065–DNA adducts (Figure 10) showing inhibition of cleavage over a 12–13-bp region and enhancements 8 bp to the 5′ side of the covalently modified adenines are quite similar and therefore do not provide a rationale for the differential recognition of these CC-1065–DNA adducts by the UVRABC nucleases. It is possible that the UVRABC nucleases are able to recognize differential features among the various CC-1065–DNA adducts that are not detected by DNase I. Alternatively, a property associated with sequence independent of the CC-1065–DNA adduct is critical for UVRABC recognition. Lastly, it is possible that because of the considerable helix-stabilizing property of CC-1065, which consequently inhibits nuclease activity, that UVRABC nuclease only cleave the subpopulation of (*Msp*I–*Bst*NI) 117-bp fragments that have a single CC-1065–DNA adduct. If this is the case, then the predominant CC-1065 single adduct formed in the 117-bp fragment will be located at A-6229 since CC-1065 has the highest affinity toward this adenine in this particular fragment sequence. At this time we cannot differentiate these possibilities. However, our results illuminate one of the dangers of interpretation of results that rely solely on multiple carcinogen modified or drug-modified DNA species.

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Expression of Human DNA Polymerase β in *Escherichia coli* and Characterization of the Recombinant Enzyme

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ABSTRACT: The coding region of a human β-polymerase cDNA, predicting a 335 amino acid protein, was subcloned in the *Escherichia coli* expression plasmid pRC23. After induction of transformed cells, the crude soluble extract was found to contain a new protein immunoreactive with β-polymerase antibody and corresponding in size to the protein deduced from the cDNA. This protein was purified in a yield of 1–2 mg/50 g of cells. The recombinant protein had about the same DNA polymerase specific activity as β-polymerase purified from mammalian tissues, and template-primer specificity and immunological properties of the recombinant polymerase were similar to those of natural β-polymerases. The purified enzyme was free of nuclease activity. We studied detailed catalytic properties of the recombinant β-polymerase using defined template-primer systems. The results indicate that this β-polymerase is essentially identical with natural β-polymerases. The recombinant enzyme is distributive in mode of synthesis and is capable of detecting changes in the integrity of the single-stranded template, such as methylated bases and a double-stranded region. The enzyme recognizes a template region four to seven bases downstream of the primer 3′ end and utilizes alternative primers if this downstream template region is double stranded. The enzyme is unable to synthesize past methylated bases N^3-methyl-dT or O^6-methyl-dG.

DNA polymerase β, a DNA repair polymerase of eukaryotic cells [for a review, see Fry and Loeb (1986)], is seen as a model enzyme for structure–function analysis of the nucleotidyltransferase reaction by DNA polymerases (Tanabe et al., 1979). This enzyme is the simplest DNA polymerase known in both size and catalytic repertoire. The human and rat enzymes are polypeptides of 335 amino acids, and secondary structure predictions suggest ordinary globular structure with high α-helix content (Zmudzka et al., 1986; SenGupta et al., 1986). The purified enzyme lacks exonuclease activities and detectable reverse reactions (Tanabe et al., 1979; Fry, 1983), and the polymerase activity is fully distributive under most reaction conditions (Detera et al., 1981). Thus, the β-polymerase mechanism is a two-substrate–two-product reaction and follows ordered Bi-Bi kinetics (Tanabe et al., 1979).

To examine physical biochemical properties and structure–function relationships of mammalian β-polymerase, we overexpressed the coding region of a human β-polymerase cDNA (SenGupta et al., 1986) in the λP_L promoter-based bacterial expression system pRC23 (Crowl et al., 1985). Here, we report the purification of the recombinant enzyme in milligram quantities. Enzymatic studies revealed that the enzyme is a characteristic β-polymerase and is appropriate for structure–function studies of this enzyme.

Materials and Methods

Materials. [γ-^{32}P]ATP was obtained from New England Nuclear or ICN Radiochemicals. Homogeneous Klenow fragment from *Escherichia coli* DNA polymerase I was a generous gift from W. E. Brown. DNA polymerase β from chick embryo was purified as described (Yamaguchi et al., 1980) and was a generous gift from A. Matsukage. β-Polymerases from HeLa cells (Mosbaugh & Linn, 1983) and rat Novikoff hepatoma (Stalker et al., 1976) were purified as previously described and were generous gifts from D. W. Mosbaugh. Preparation of mouse and calf thymus DNA polymerases β and Western blotting materials were as described earlier (Swack et al., 1985; Tanabe et al., 1979; Karawya et al., 1984). T4 polynucleotide kinase, T4 DNA ligase,