A Comparison of the Rates of Reaction and Function of UVRB in UVRABC- and UVRAB-mediated Anthramycin-N2-Guanine-DNA Repair*

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The repair of anthramycin-DNA adducts by the UVR proteins in Escherichia coli follows two pathways: the adducts may be incised by the combined actions of UVRA, UVRB, and UVRC, or alternatively, the anthramycin may be removed by UVRB and UVRB in the absence of UVRC and with no DNA strand incision. To assess the competition between these two competing pathways, the rate of UVRABC-mediated excision repair of anthramycin-N2-guanine DNA adducts and the rate of UVRB-mediated removal of the adduct were measured with single end-labeled DNAs under identical reaction conditions. UVR protein concentrations of 15 nM UVRA, 100 nM UVRB, and 10 nM UVRC protein were chosen to mimic in vitro concentrations. With these UVR protein concentrations and anthramycin-DNA concentrations of 1–2 nM the incision reaction and the release reactions are described by first-order kinetics. The rate of the UVRABC reaction, measured as the increase in incised fragments, was six to seven times faster than the rate of the UVRAB reaction, measured as the decrease in incised fragments. The UVRABC reaction rates on anthramycin-modified linear DNA was four to five times the incision rate measured on the same DNA irradiated with ultraviolet light. We also investigated the role of the ATPase function of UVRB in UVRABC-mediated anthramycin removal. We found that a UVRB analogue with alanine at arginine 51, which retains near wild type ATPase activity, supported removal of anthramycin in the presence of UVRA, whereas a UVRB analogue with alanine at lysine 45, which abolishes the ATPase activity, did not. UVRB*, a specific proteolytic cleavage product of UVRB which retains the ATPase activity, did support removal of anthramycin in the presence of UVRA.

The UVRABC enzyme complex is responsible for the initial steps in nucleotide excision repair in Escherichia coli. Repair by the UVRABC proteins shows a broad specificity for many types of DNA damage induced by a variety of chemical agents and UV irradiation (for review see Van Houten (1990), Grossman and Yeung (1990), and Sancar and Sancar and (1988)). In all these cases the UVRABC mechanism of repair is through incision near the lesion and is completely dependent on the combined actions of all three proteins; no incisions are produced with single proteins or any pairwise combination. We have recently reported a singular exception to the generality that incision always accompanies repair. Anthramycin-N2-guanine adducts which have the unique feature of stabilizing the DNA helix (Hurley and Needham VanDeVanter, 1986), although subject to the incision pathway as noted above (Walter et al., 1988), are also repaired by the UVRA and UVRB proteins, in vitro and in vivo, without incision of the DNA. This reaction of the UVRA and UVRB proteins was limited to anthramycin-DNA adducts and does not occur with UV induced photoproducts. In the presence of UVRAB and the absence of UVRC anthramycin-DNA is repaired by a second pathway involving the direct release of anthramycin without concomitant incision or the production of apurinic sites (Tang et al., 1991). In vivo, the significance of UVRAB-mediated repair is evidenced by the higher transfectivity of anthramycin-modified φX174 in worC− mutant cells than in wild type cells and the lower transfectivity of the anthramycin-modified φX174 DNA in worA− and worB− mutant cells (Tang et al., 1991).

Anthramycin, isolated from Streptomyces rufusinues, is a pyrrole (1,4)-benzodiazepine antibiotic, with potent anti-tumor activity (Hurley et al., 1986). It binds specifically to the exocyclic 2-amino group of guanine in double-stranded DNA forming a covalent anthramycin-DNA adduct (Hertzberg et al., 1986; Hurley et al., 1977). The anthramycin-DNA adducts lie within the minor groove of the DNA double helix, and this structure results in little or no distortion of the DNA helix structure. DNA with covalent anthramycin adducts displays a decreased sensitivity to S1 nuclease and an increase in melting temperature (Hurley and Thurston, 1984). Anthramycin shows a DNA sequence specificity for adduct formation; studies on defined DNA sequences indicate that it bonds preferentially to guanines in the triplets purine Guanine purine (Walter et al., 1988; Hertzberg et al., 1986).

Repair initiated by UVRABC involves a series of sequential steps. Damage recognition may be initiated by binding of the UVRA subunit which can discriminate between damaged and undamaged bases (Van Houten et al., 1988; Mazur and Grossman, 1991). UVRA, which does not bind to DNA by itself, interacts with the UVRA-DNA complex to form a UVRAB-DNA complex (Caron and Grossman, 1988; Orren and Sancar, 1989). Alternatively, the UVRAB complex may form directly in solution and interact with DNA to find a damaged base (Bertrand-Burggraf et al., 1991). In either case a UVRAB-damaged DNA complex results with altered DNA structure. Here is evidence for the release of UVRA from the complex

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at this point (Orren and Sancar, 1990). The remaining UVR(A)B protein-DNA complex interacts with UVRC to produce the characteristic incisions.

We have found that UVRAB mediated anthramycin release to be an almost requirement for both the UVRB and UVRBC proteins and that this mechanism is capable of removing all of the covalently bound anthramycin from a linear DNA in the absence of UVRB. On the other hand, the reaction with all three UVR proteins shows incision of anthramycin-modified DNA to be easily measurable, suggesting that the UVRABC incision reaction dominates over UVRAB-mediated anthramycin release. These observations raise an important question: What is the rate of UVRAB-mediated anthramycin release in comparison with the UVRABC incision rate? Is the UVRAB reaction a slow process outrun by a rapid UVRABC incision at most anthramycin-DNA sites? In reaction mixtures containing all three UVR proteins, both UVRABC incision and UVRAB release of anthramycin at anthramycin modification sites in DNA would be expected to occur. This competition is assessed in this report by comparing the rates of the two reactions in vitro.

We have found UVRAB-mediated anthramycin release to require ATP (Tang et al. 1991). The role of ATP in UVRABC reactions is complex. UVRB is an ATPase, and ATP facilitates UVRB binding to damaged DNA. Interestingly a putative ATP binding domain is also present in the UVRB sequence, and furthermore, UVRB exhibits a cryptic ATPase which may be activated upon DNA binding (Caron and Grossman, 1988). The role of the UVRB ATPase in UVRB-mediated anthramycin release is investigated by comparing the release of anthramycin in the UVRB reaction with the release in the presence of two UVRB mutant proteins with altered ATP binding domains (Seeley and Grossman, 1989) and UVRB*, a specific proteolytic fragment of UVRB which retains the ATPase function. These experiments allow us to assess the quantitative significance of UVRB-mediated release of anthramycin and to determine more exactly the role of UVRB in the reaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anthramycin was obtained from the drug repository of the National Cancer Institute. Restriction enzymes HaeIII and Hindl were purchased from Bethesda Research Laboratories. Restriction enzyme BstNI was from New England BioLabs. Yeast tRNA was obtained from Sigma. All other chemicals were reagent grade or better. Nucleotide-containing [32P]cTPP was obtained from Amersham Corp. or Du Pont-New England Nuclear.

**DNA Fragments**—Plasmid pBR322 was isolated and purified by cesium chloride density gradient centrifugation. The BstNI-BstNI 383-bp DNA fragment was isolated by agarose gel purification with minimal exposure to UV light. The fragment was 3' end labeled with [α-32P]cTPP using DNA polymerase I (Klenow fragment) and purified by centrifugation through Sephadex G-50 in 10 mM Tris, pH 7.5, 10 mM EDTA. The fragment was phenol-chloroform extracted and ethanol precipitated. A typical labeling experiment used 1.75 ng (7 pmol of DNA) and yielded 100,000 cpm/pmol-end (Cherenkov counting). After a second enzyme digestion with HindIII the BstNI-Hind III 247-bp and HindIII-BstNI 140-bp fragments were purified from a 5% polyacrylamide gel.

**Anthramycin Modification of Labeled DNA**—Stock solutions of anthramycin were prepared in methanol and stored at −20°C. The concentration was determined by absorbance at 333 nm using a molar extinction coefficient of 36,800 (Hurley et al., 1977). Dilutions in methanol were used for modification. To achieve different degrees of anthramycin modification a fixed volume (10 μl) of different concentrations of anthramycin solution was added to constant amounts of DNA (0.2 μg, in 50 μl of 10 mM Tris-HCl, pH 7.5, 1.0 mM EDTA (TE)). The reaction proceeded at room temperature for 2 h and was stopped by the addition of ammonium acetate (to 1.0 M) and 2.5 volumes of ethanol. The unbound anthramycin was removed by centrifugation, and the DNA pellet was washed with 0.8 ml of 80% ethanol and dissolved in TE.

**UVRABC Nuclease Reactions**—UVRB, UVRB, and UVRB proteins were purified from E. coli K12 strain CH296 carrying plasmids pUNC45 (uorA), pUNC211 (uorB), or pDNR229 (uorC) as previously described (Caron and Grossman, 1988). Two UVRB mutants containing substitutions of alanines at residues 45 (K45A) and alanines 51 (N51A) were purified as described (Seeley and Grossman, 1989). An aliquot of modified labeled DNA (usually 10,000 cpm, for a final DNA concentration of 0.6-2.0 nm) was reacted in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 100 mM KCl, 1 mM ATP, and 1 mM dithiothreitol, UVRB, UVRB, and UVRB proteins were added giving a final concentration of 15 nM each or as indicated in a final volume of 25 μl. The UVR proteins were dissolved in 50 mM Tris-HCl, pH 7.5, 50% glycerol, 0.1 mM dithiothreitol, 0.1 mM EDTA and diluted in the same buffer as required. Reaction times were as indicated at 37°C. Reaction was terminated with stop buffer to give final concentrations of 1.0 M, 7.5 mM MgCl2, 1 mM ammonium acetate, and 50 μg/ml RNA. After phenol-chloroform extraction (phenol:chloroform:isoamylalcohol, 1:96:0.04) and chloroform extraction the DNA was precipitated with 2.5 volumes of ethanol, washed with 80% ethanol, dried, and dissolved in 10 mM NaOH, tracking dye mixed.

**UVRABC Reactions—** Different amounts of anthramycin-DNA adducts by UVRAB was assayed by the reduction in UVRABC incisions on end-labeled anthramycin-modified DNA after reaction of the modified DNA with UVRABC. Two methods were used. In Method 1, UVRABC reactions were identical to the UVRABC reactions described above except for the absence of UVRB. The DNA was isolated from the reaction mixture as above and subjected to a second reaction under the same conditions which included all three UVR proteins. Recovery of DNA was typically >90%. In Method 2, UVRABC reaction conditions were again identical to the UVRABC reactions but without UVRB. The remaining UVRABC-sensitive sites on the modified DNA were determined by the direct addition of UVRB to the UVRABC reaction. The possibility of depletion of ATP over the time course of the UVRABC reaction, which would lead to an artifically low incision level, was checked by comparing the level of incisions found when either UVRB alone or UVRB and supplemental ATP (for a final concentration of 1 mM ATP) were added to the UVRABC reaction. No difference in incision level was found between the two methods. Additionally, UVRABC reactions were run with unmodified DNA (which stimulates the activity of the UVRB ATPase as well) for 90 min following which anthramycin-modified DNA and UVRABC were added. The level of incisions found in this reaction was identical to the level measured directly with the modified DNA. We conclude that the depletion of ATP by the ATPase activity of UVRB or inactivation of the UVRB or UVRB proteins over the course of an assay is not an impediment to using Method 2.

**UVRABC Kinetic Analysis—** For UVRABC reactions, anthramycin-modified DNA was preincubated for 5 min with 15 nM UVRA and 100 nM UVRB at 37°C in reaction buffer, and the reaction was started with the introduction of UVRC at the indicated concentrations. UVRABC reactions were sampled at time intervals determined in preliminary experiments to encompass at least 90% of the total intensity change, and then at a time corresponding to completion of the reaction, the DNA fragments were separated on sequencing gels, and the intensities of the bands were quantified. For UVRABC reactions, the modified DNA was preincubated for 5 min with 15 nM UVRA, and reaction buffer was added upon addition of UVRB to 100 nM or as indicated at 37°C. Aliquots were removed at times determined by preliminary experiments, and the reactions were processed as for Method 1 above or alternatively for Method 2. DNA fragment intensities were measured at individual incision sites, and the intensity of the fragments at each time point was used to indicate the extent of incision for UVRABC or remaining anthramycin-DNA for UVRAB reactions. Semilogarithmic plots were constructed for UVRABC-mediated anthramycin release reactions of the remaining UVRABC nuclease at different substrate concentrations versus time and for UVRABC incision reactions of the difference between the intensities of bands at the completion of the reaction (I) and the intensity at time t (J) versus time (Jencks, 1969). Comparisons of the rates of incision and release at individual sites were similarly made. Apparent first order rate constants and standard rates were determined by semilogarithmic plots of the data.
errors were derived by linear regression.

UV Irradiation—A 200-μl aliquot of end-labeled DNA was irradiated, and aliquots were removed after exposures of 0, 600, 1,200, 2,400 or 3,600 J m⁻² of UV light with a fluence rate of 3.5 J m⁻² s⁻¹ at room temperature (Westinghouse germicidal lamp, R15TR. major emissions at 254 nm).

T4 Endonuclease Reactions—T4 endonuclease V (1.2–4 μg) prepared by the method of Friedberg et al. (1980) was incubated with 10 ng of end-labeled DNA in 1 mM EDTA, 0.1 M NaCl, 5 mM Tris-HCl, pH 7.7 (final volume 25 μl), at 37 °C for 60 min. The reaction was stopped, extracted, and ethanol precipitated as above for UVRABC reactions.

Sequencing Reactions and Quantitation of Band Intensity—Chemical sequencing as described by Maxam and Gilbert (1980) was employed with the modifications described (Pierce et al., 1989). Sequencing gels were 8% acrylamide in 7 M urea, 50 mM Tris-HCl, 50 mM borate, and 10 mM EDTA, pH 8.3. The gels were dried in a Bio-Rad gel dryer and exposed to Kodak X-Omat RP films at −70 °C. Several exposures were made to avoid saturated bands. The intensity of bands was determined by Bio-Image Analyzer. For some experiments the intensities were also determined by PhosphorImager Analysis directly from the dried gels. Similar results were obtained with either method.

RESULTS

Rate of the UVRABC Reaction with Anthramycin-N2-Guanine-DNA—It has been shown that in the absence of DNA repair synthesis and the subsequent ligation, the UVR(A)BC nuclease remains bound to the damaged DNA region after incision; therefore, the UVRABC nuclease incision reaction is a stoichiometric rather than a catalytic reaction (Husain et al., 1985; Caron et al., 1985). Since the exact composition of the incision complex is not known, we have chosen UVR protein concentrations which mimic the estimated in vivo concentrations for determining the rate of UVRABC incision on the anthramycin-modified DNA substrate; that is, 15–30 nM for UVRA, 100–700 nM for UVRR, and 0.5–15 nM for UVRC (Sancar and Sancar, 1988) and DNA concentrations which result in large excesses of UVR proteins. The rate of the UVRABC reaction was followed by the appearance of the characteristic DNA fragments produced from anthramycin-modified 3' end-labeled DNA fragments incubated with UVR proteins. The typical results are in Fig. 1A; UVRABC incision of anthramycin-modified Hinfl-BstNI 247-bp DNA resulted in eight major bands (U1–U6, U9, U10) at -AGA- or -AGG-sites and two minor bands at -TGA- sites (U7 and U8) with the intensity at each site increasing with incubation time. The UVRABC rate was determined from autoradiographic intensity measurements at incision sites in this DNA and also from a BstNI-Hinfl 140-bp DNA, which showed two major anthramycin modifications attributable to -AGA- sites. To explore any DNA sequence effects the rate at each incision site as well as on a complete fragment basis were calculated. Fig. 1B shows the results on a complete fragment basis. With fixed UVRA (15 nM) and UVRR (100 nM) concentrations the extent of incision was essentially the same with initial UVRABC concentrations from 2 to 15 nM. With the lower UVRABC concentration of 1 nM the incision level decreased to less than half the value seen at the higher UVRABC concentrations. Considering the lack of turnover of the UVRABC proteins this result suggests that UVRC is either stoichiometric or in excess at concentrations above 2 nM in these reactions.

In Fig. 2, the results after the UVRABC incision reaction with the 247-bp DNA ([UVRC] = 10 nM) are plotted. The semilogarithmic plot (Fig. 2, inset) is linear for at least 8 half-lives and allows us to conclude that the reaction is pseudo-first order. The data were fitted to ln(I/I₀) = -kt, where I is the intensity at the end of the reaction and I₀ the intensity at t, and the apparent first order rate constant kₐₜₙ,ABC,ATm = 0.79 ± 0.03 min⁻¹ was estimated by linear regression. Rate determinations at other UVRABC concentrations were similarly made (Fig. 3). Data for the UVRABC incision rate at different UVR concentrations indicate that the pseudo-first order rate constant increases with increasing [UVRC] and reaches a plateau near kₐₜₙ,ABC,ATm = 0.8 min⁻¹. The dependence of the first order rate constant on the UVRC concentration indicates that the reaction is pseudo-second order overall.

Since the UVRABC reaction is stoichiometric, the limiting incision level found at UVRC concentrations above 2 nM could be a consequence of limiting amounts of active UVRA or UVRB in spite of their presence in calculated excess. In Fig. 4, UVRABC incision levels were measured at a fixed UVRC concentration (15 nM) and with varying UVRA (15–30 nM) and UVRB (100–700 nM) concentrations. No change
in the incision level with these higher protein concentrations is evident, nor is there an appearance of nonspecific DNA fragments which would interfere with the analysis.

Apparent variations in the UVRABC incision rate for different bands of up to 2-fold were seen, but their significance is unclear. The rates of incision of individual bands showed no correlation with the extent of incision.

Kinetics of UVRAB Removal of Anthramycin—The rate of anthramycin removal by UVRAB was determined indirectly by measuring the decrease in UVRABC nuclease-sensitive sites following the pretreatment of anthramycin-modified DNA with UVRA and UVRB proteins. This has the experimental advantage that a direct comparison of the decrease in UVRABC nuclease-sensitive sites are reasonably linear and yield the apparent first order rate constant $k_{AB} = 0.076 \pm 0.005 \text{ min}^{-1}$. Varying the UVRB concentration in the range of 15–100 nM yielded rates of UVRABC-mediated anthramycin release varying from $k_{AB} = 0.05–0.13 \text{ min}^{-1}$ with an average of $k_{AB} = 0.092 \pm 0.012$ (mean ± S.E. from seven determinations, data for 247- and 140-bp DNAs). The differences in rate show no relation to the concentration of UVRB and probably represent experimental error. The lack of dependence of the first order rate of UVRABC-mediated anthramycin release on UVRB concent-

FIG. 2. Rate of the UVRABC incision on anthramycin-modified linear DNA. The rate of UVRABC incision was determined by densitometry of autoradiograms similar to Fig. 1 except with UVRA, UVRB, and UVRC concentrations of 15, 100, and 10 nM, respectively. The sum of the intensities for all the marked bands of Fig. 1 at each time point $(I)$ is plotted versus time. Semilogarithmic plotting of the sum of intensities $(I)$ at the end of the UVRABC reaction (15 min) minus the intensity at time $t$ $(I_t, \text{inset})$; see “Experimental Procedures” gives a first order rate constant, $k_{obs,ABC,Atm} = 0.79 \pm 0.02 \text{ min}^{-1}$ (correlation coefficient, $r^2 = 0.98$). The DNA was 0.6 nM, and 1.2 anthramycin adducts/DNA was estimated from Poisson statistics on the uncut full-length DNA.

FIG. 3. The relationship of the UVRABC incision rate constant to UVRC concentration. The first order rate constants for UVRABC incision ($k_{obs,ABC,Atm} \text{ min}^{-1}$) were determined as in Fig. 2 with different UVRC concentrations and are plotted versus the UVRC concentration (nM). The rate of UVRABC-mediated anthramycin release (see Fig. 5) is plotted at UVRC = 0.

FIG. 4. The effect of UVRA and UVRB concentrations on the UVRABC incision of anthramycin-modified DNA. HinfI-BstNI 247-bp 3′ end-labeled DNAs with (lanes 1–6 and lanes 14–16) or without (lanes 7–13) modification with 200 pmol of anthramycin were reacted with different concentrations of UVR proteins as indicated on the top of the figure for 40 min at 37°C. The anthramycin-induced UVRABC incision bands (U1–U10) are depicted at the side of the panel. Lanes 7–10 are Maxam and Gilbert sequencing reactions.

transfectivity of modified DNA and is, then, a sensitive measure of UVRAB removal of anthramycin from DNA (Tang et al., 1991).

The anthramycin-modified HinfI-BstNI 247-bp substrate was incubated with 15 nM UVRA in reaction buffer for 5 min. The UVRAB reaction was initiated with the addition of UVRB. Fig. 5A shows a typical experiment with [UVRB] = 100 nM. The decrease in the sum of intensities of the eight major modification sites is plotted in Fig. 5B. Semilogarithmic plots (Fig. 5B, inset) of the decrease in UVRABC nuclease-sensitive sites are reasonably linear and yield the apparent first order rate constant $k_{AB} = 0.076 \pm 0.005 \text{ min}^{-1}$. Varying the UVRB concentration in the range of 15–100 nM yielded rates of UVRABC-mediated anthramycin release varying from $k_{AB} = 0.05–0.13 \text{ min}^{-1}$ with an average of $k_{AB} = 0.092 \pm 0.012$ (mean ± S.E. from seven determinations, data for 247- and 140-bp DNAs). The differences in rate show no relation to the concentration of UVRB and probably represent experimental error. The lack of dependence of the first order rate of UVRABC-mediated anthramycin release on UVRB concen-

Rate of UVRAB and UVRABC Repair of Anthramycin-DNA Adducts 24719
Rate of UVRABC Reaction on UV Irradiated DNA—The rate of UVRABC incision of UV photoproducts on the same 247-bp DNA is of interest since UVRABC alone does not reduce the number of pyrimidine dimers present in the DNA, allowing a measurement of the UVRABC incision rate without a competing reaction (Tang et al., 1991). It was reported recently that the efficiency of UVRABC incision at some site-directed pyrimidine dimers was low with approximately 20% of the total sites incised (Bertrand-Burggraf et al., 1991). To estimate the efficiency of UVRABC incision of the UV irradiated 247-bp DNA the DNA fragment intensities produced by UVRABC incision were compared with those produced by T4 endonuclease V. Densitometry of the autoradiograph of the experiment shown as Fig. 6B confirms that UVRABC and T4 endonuclease V produced similar increasing levels of incision on DNA irradiated with UV doses calculated to produce

**Fig. 5.** Time course of the decrease in UVRABC-sensitive sites in the anthramycin-modified DNA fragments after UVRAB treatment. Panel A, the 3' end-labeled 247-bp HindIII-BstNI DNA fragment (0.6 nM) was modified with 200 pmol of anthramycin (1.2 anthramycin/DNA), pretreated with UVRAB, and then the UVRABC nuclease-sensitive sites were detected. The UVRAB reactions were by Method 1 (see “Experimental Procedures”), and the DNA was preincubated for 5 min at 37°C with 15 nM UVRAB in reaction buffer following which the reactions were started by the addition of UVRB to 100 nM. Aliquots were taken at the times indicated at the top of the figure for lanes 8-23 and after isolation the DNA was subjected to UVRABC digestion with 15 nM UVRA, 100 nM UVRB, and 15 nM UVRC for 40 min at 37°C. The resultant DNA was separated on a sequencing gel, and a typical autoradiogram is shown. Lanes 1-4 are Maxam and Gilbert sequencing reactions. Control reactions contained only UVRA (lane 5, 15 nM) or UVRB (lane 6, 100 nM) in the first reaction which was followed by a second UVRABC detection reaction as above. Lane 7 is the result of UVRABC digestion without a second reaction, and lane 24 is the result of UVRABC reaction using the second reaction enzymes with modified DNA. Panel B, summed DNA fragment intensities measured by densitometry of the autoradiogram from the above experiment and normalized to the intensity at t = 0 are plotted versus time. Inset, a semilogarithmic plot (see "Experimental Procedures") of the same data is shown and yields the apparent first order rate constant, kAB = 0.08 ± 0.01 min⁻¹.

**Fig. 6.** Panel A, time course of UVRABC incision of UV irradiated 247-bp DNA. The DNA was irradiated with UV at 1,200 J/m² and reacted with UVRABC (UVRA 15 nM, UVRB 100 nM, and UVRC 8 nM) according to "Experimental Procedures." An autoradiogram of a sequencing gel showing the progress of the reactions is shown. Reaction times were 0.25, 0.5, 1, 2, 4, 6, 8, 12, 16, 20, 25, and 30 min for lanes 1-12, respectively. Maxam and Gilbert sequencing reactions are labeled G, GA, GC, and C. Panel B, comparison of the extent of incision of UV irradiated 247-bp DNA by UVRABC and T4 endonuclease. The 247-bp HindIII-BstNI DNA fragment was irradiated with different UV fluences (labeled 1X, 2X, 4X, and 6X) with 1X representing 600 J/m² (see "Experimental Procedures"). The DNA of lanes marked T4 Endo was incised with 1.2 µg of T4 endonuclease V, and the DNA of lanes marked UVRABC was incised with UVRABC (UVRA = 15 nM, UVRB = 100 nM, and UVRC = 15 nM) and the products separated on a sequencing gel. Sites labeled with T on the right correspond to photodimers produced at consecutive thymines which show more intense bands after T4 endonuclease V reaction than after UVRABC reaction. Sites indicated on the right labeled with U correspond to UVRABC incision sites which are more intense than T4 endonuclease V incisions. The brackets encompass the bands used for kinetic analysis.
One to six dimers/strand. It is worth noting that at two thymine-thymine sites the extent of UVRABC reaction was smaller than the extent of T4 endonuclease V reaction, whereas at two cytosine-cytosine sites UVRABC incisions are much stronger than the T4 endonuclease V incisions (compare the T4 Endo lane 6x with UVRABC lane 6x in Fig. 6B). Reactions with twice and four times the amount of T4 endonuclease V or UVRABC proteins showed no further increase in incision levels (data not shown).

The time course of UVRABC incision on UV irradiated 247-bp DNA was determined as detailed for anthramycin-modified DNA at a UVEC concentration of 8 nM, and the results are shown in Fig. 6A. The quantitative analysis was confined to a length of DNA containing 19 sites which were more sensitive to UVRABC incision than T4 endonuclease incision. The close correspondence of the individual site rates and the overall rate for incision of anthramycin-DNA suggest that this procedure will give a good estimate of the UV incision rate. Fig. 7 is a plot of the combined intensity of the bands corresponding to these sites as an increase in UVRABC nuclease-sensitive sites versus time. Semilogarithmic plotting of these data (Fig. 7, inset) suggests that under these conditions UVRABC incision of UV irradiated DNA is a first order reaction with a half-life of reaction of 6 min and allows us to calculate an apparent first order rate constant of \( k_{\text{ob,ABC,UV}} = 0.16 \pm 0.03 \text{ min}^{-1} \).

The UVRAB- and UVRABC-mediated Reaction with Two Mutant UVRB Proteins and UVRB*—Two UVRB proteins UVRB-K45A and UVRB-N51A, prepared by site-directed mutagenesis of UVRB, were tested for their ability to participate in UVRABC-mediated anthramycin removal. UVRB-K45A is a UVRB protein with alanine substituted for lysine at amino acid position 45, and UVRB-N51A is a UVRB with alanine substituted for arginine at position 51 (Seeley and Grossman, 1989). Substitution of alanine for lysine in the putative ATP binding site of UVRB at position 45 results in a protein inactive in complementing UVRABC repair of UV damage in \( uvrB^- \) cells, whereas substitution at the nearby arginine 51, results in a UVRB protein with nearly normal activity (Seeley and Grossman, 1990). UVRB*, which lacks the 40 carboxyl-terminal amino acids of UVRB, is a single-stranded DNA-dependent ATPase with greatly diminished incision activity in the presence of UVRABC and UVRA (Caron and Grossman, 1998).

The functions of UVRB*, UVRB-K45A, and UVRB-N51A in the UVRABC-mediated incision on anthramycin-modified DNA substrates are shown in Figs. 8 and 9. N51A is active in UVRABC incision of anthramycin-modified DNA and K45A completely inactive (Fig. 8, lanes 22 and 18). These results demonstrate the activity of the N51A and K45A protein in incising anthramycin-DNA adducts to correspond directly to their reported ability to complement repair of UV damage and incise UV damaged supercoiled DNA. UVRABC* also produces incisions with this DNA (Fig. 8, lanes 13, and Fig. 9, lane 20), although the extent of reaction is lower than with UVRABC (compare Fig. 9, lanes 19 and 20). The activity of UVRB* with the 247-bp HinfI-BstNI pBR322 DNA was less than one-tenth the activity found with UVRB (results not shown).

The ability of these three proteins to participate with UVRA in UVRABC-mediated anthramycin release was tested on the same DNA, and the results are shown in Fig. 8. No decrease in UVRABC nuclease-sensitive sites was found in a reaction with the UVRB-K45A by itself or in the presence of UVRA. In contrast, a decrease in UVRABC nuclease-sensitive sites was observed in the reaction with UVRA+UVRB-N51A+UVRB or UVRR*+UVRA with no decrease in UVRABC nuclease-sensitive sites in the absence of UVRA. Anthramycin release in the presence of 15 nM UVRA and UVRB-N51A, UVRB*, or wild type UVRB with decreasing UVRA-N51A and UVRB protein amounts allows us to estimate the UVRA-N51A analogue to be 80% as active as wild type UVRB (compare lanes 14 and 15 with lanes 2 and 3 in Fig. 9). UVRB* (Fig. 9, lanes 8–12) is as active as UVRB. The purity of UVRR*, UVRA, and the mutant UVRB is demonstrated by the results of SDS-polyacrylamide gel electrophoresis (Fig. 10). UVRB* is clearly separated from UVRB, and the UVRR* protein shows no contamination with UVRB. These results indicate that the ability of UVRR* to participate in UVRABC incision and UVRABC-mediated anthramycin removal is not caused by the presence of UVRB.

**DISCUSSION**

We have reported previously that there are two pathways mediated by UVR proteins which lead to repair of anthramycin lesions (Tang et al., 1991). First, anthramycin-modified DNA is a substrate for the UVRABC complex resulting in incisions 6-8 bp 5' and 3 or 4 bp 3' to the modified base (Waller et al., 1988). Second, in the absence of UVRA, reaction with UVRB results in the direct release of anthramycin from anthramycin-N2- guanine-DNA adducts possibly through the reversal of the modification reaction to yield...
unmodified DNA (Tang et al., 1991). Our findings raise two immediate questions: 1) In vivo are the anthracycin-DNA adducts repaired only by the UVRAB-mediated or UVRABC incision pathway or by both? and 2) To what extent does the UVRAB reaction with anthracycin-DNA, by removing anthracycin directly, lead to an underestimate of anthracycin modification as determined by the extent of UVRABC incision? In this paper we attempted to answer these two questions by investigating the rate of UVRABC-mediated anthracycin release and the rate of UVRABC incision on anthracycin-modified DNA under conditions in which the UVR protein concentrations are in excess to the modified DNA and similar to in vivo levels.

The overall reaction of UVRABC proteins with anthracycin-modified DNA may be represented as sequential reactions beginning with protein complex formation at the damaged site
mycin release in the presence of only the UVRA and UVRB proteins, as we have shown to occur,

\[ \text{Atm-DNA} - (A_3)_B \xrightarrow{k_{\text{RA}} \text{ UVRA}} \text{Atm + DNA}(A_3)_B \]  
(Eq. 2)

and the second is the UVRABC anthramycin-DNA incision reaction in the absence of UVRAB-mediated anthramycin release,

\[ \text{Atm-DNA} - (A_3)_B + \text{UVRC} \xrightarrow{k_{\text{ABC}} \text{ UVRABC}} \text{Atm-DNA}(A_3)_B \text{UVRC} \]  
(Eq. 3)

where incised DNA retaining UVR proteins has the subscript i and the parentheses illustrate that the composition of the incision complex and the UVRABC-DNA complex are not known with certainty and may or may not include the UVRA protein (Orren and Sancar, 1990). Additionally, as indicated in Equation 2, DNA may still be complexed with UVRA and UVRB after anthramycin release.

Both of the mechanisms 2 and 3 have in common the reaction of the anthramycin-DNA-UVRA protein complex and at least formally could proceed as concurrent reactions during UVRABC incision of anthramycin-modified DNA. The UVRABC-anthramycin-DNA incision reaction rate was studied with UVRA and UVRB proteins in 10-fold or greater excess to the modified DNA and was second order overall and first order at any particular UVRC concentration, in agreement with Equation 3 above. The observed pseudo-first order rate constant for the incision reaction, \( k_{\text{obs,ABC,Atm}} \), was found to vary with UVRC concentration but to reach plateau values near 0.8 \( \text{min}^{-1} \) at UVRC concentrations in excess of 10 nM. The actual maximum rate may be lower since little variation in the rate is seen with 2 \( \leq \text{UVRC} \leq 12 \) nM, where the average rate is 0.61 \( \pm 0.05 \text{ min}^{-1} \). The rate of UVRABC-mediated anthramycin release from anthramycin-modified DNA was determined to be described by a pseudo-first order rate constant of \( k_{\text{RA}} = 0.09 \pm 0.01 \text{ min}^{-1} \). Therefore, the overall UVRABC-anthramycin-DNA reaction rate, \( k_{\text{obs,ABC,Atm}} \), is six to eight times greater than the UVRAB-mediated anthramycin release rate. If the separate reactions of incision by UVRABC and anthramycin release by UVRAB proceed in parallel in the presence of all three UVR proteins then their first order rate constants may be added to obtain \( k_{\text{obs,ABC,Atm}} \) (Jencks, 1969). This leads to an estimate of 0.5–0.7 \( \text{min}^{-1} \) for the rate of the incision reaction in the absence of the release reaction.

Since the products of concurrent first order reactions are produced in the same ratio as the rate constants, these results suggest as a rough approximation, in the presence of all three UVR proteins, \( \text{in vitro} \), approximately 80–85% of the anthramycin-modified DNA is incised by UVRABC with the remainder repaired by anthramycin release. Extrapolations of these data \( \text{in vivo} \) conditions must consider the high DNA concentration \( \text{in vivo} \) as compared with the low concentration used in these \( \text{in vitro} \) measurements. However, the rates of incision or anthramycin release measured here apply to preformed UVRAB-DNA complexes, the presumed intermediate in the incision reaction, which may be formed at different rates \( \text{in vivo} \) but once formed are subject either to decay by loss of anthramycin or interaction with UVRC leading to incision \( \text{in vivo} \). With these assumptions, considerations similar to those discussed above for the \( \text{in vitro} \) case apply and suggest that 80–85% of the complexes will be subject to incision and the remainder undergo release of anthramycin.

Reaction of anthramycin-DNA with high concentrations of UVR proteins may result in the anthramycin-DNA being present primarily as complexes containing UVRC. In this case, if anthramycin is not released from these complexes or if they go on to produce incisions after anthramycin release, the rate measured for Equation 3 is the rate of incision. A study of the anthramycin release reaction under these conditions will be necessary for a full evaluation of its importance.

Under some conditions the number of UVRABC-anthramycin-DNA complexes may be elevated beyond the available UVRC- and UVRABC-mediated repair may predominate. This occurs trivially in a \( \text{uvrC} \) cell, where we have previously shown the UVRAB reaction to be sufficient for anthramycin repair (Tang et al., 1991). This may also occur when sufficient anthramycin DNA damage is inflicted to result in SOS induction. During induction, UVRB protein increases 10–100-fold, and UVRA protein increases 5–10-fold while the UVRC protein level remains relatively constant (Van Houten, 1990). Turnover of the UVRABC complex depends on the further activities of UVRD, DNA synthesis, and ligation. The slow turnover of UVRC (0.08 \( \text{min}^{-1} \); Husain et al., 1985) could result in the sequestering of UVRC, increasing the importance of the UVRABC reaction.

The finding that the rate of UVRAB repair is slower than the rate of UVRABC incision is intriguing. The preincubation of anthramycin-DNA with either UVRA alone or the combination of UVRA and UVRB followed by addition of the complementing UVR proteins results in UVRABC incision rates which are similar (data not shown). This is not the expected result if the slower rate of the UVRABC repair is determined by the rate of the UVRA-DNA and UVRC interaction. The lack of dependence of the UVRABC-mediated anthramycin removal rate on UVRC at concentrations above 15 nM suggests that UVRABC-anthramycin-DNA complex formation is rapid in comparison to anthramycin release under these conditions and that the rate determining step in UVRABC-mediated anthramycin release could be the slower hydrolysis of the anthramycin-N2-guanine bond.

The UVRABC reaction with UV irradiated DNA was also pseudo-first order under these conditions and yielded a rate of \( k_{\text{obs,CUV}} = 0.16 \pm 0.03 \text{ min}^{-1} \) with the 247-bp DNA at 8 nM UVRC. This is considerably slower than the average rate of 0.61 \( \text{min}^{-1} \) for UVABC incision of anthramycin-modified DNA and slower than the low estimate of 0.5 \( \text{min}^{-1} \) for the first order incision of anthramycin adducts in the absence of the UVRABC-mediated release reaction. We undertook this comparison to have a determination of the UVRABC incision rate, with a substrate which does not undergo a concurrent UVRABC-mediated reaction. However, the relative rates lead to the conclusion that UVRABC incision of anthramycin adducts is much faster than the corresponding incision of UV photoproducts. The competence of our UVRABC preparations is indicated by the similar overall incision levels of T4 endonuclease V and UVRABC shown in Fig. 6B. There is no information comparing the UVRABC rate of incision with various adducts; however, the extent of reaction varies widely (Van Houten, 1990). For example, the efficiency of UVRABC incision at a site-directed psoralen adduct was five times the efficiency at a site-directed pyrimidine dimer (Bertrand-Burggraf et al., 1991). We are left with the possibilities that the rate of UVRABC incision of UV photoproducts depends on the UVRA protein concentration even at the high levels used here or that the rate varies with the type of DNA damage. These results suggest that the affinity of UVRABC toward DNA may depend on the conformation of the DNA damage.

The mechanism of anthramycin release by UVRAB from
anthramycin-N2-guanine-DNA adducts is unknown. Using a DNA fragment containing a site-directed thymine dimer, Shi et al. (1992) have found that UVR(A)B-DNA binding induces a 127° kink in DNA. If UVR(A)B binding to anthramycin-DNA adducts causes a similar DNA bending, then the release of anthramycin may be because of 1) increased hydrolysis rates resulting from strain or increased accessibility at the aminic bond introduced by the bending action or 2) introduction of the acid-labile aminic bond into an acidic environment near the UVRB protein. Alternatively, UVRB may play a more direct role. The requirement for ATP and for the UVRB protein in this process allowed us to investigate the roles of protein-DNA binding and UVRB further. Two ATPases have been found in UVR proteins, one associated with UVRA and two altered UVRB proteins, N51A and UVRB*, have the same activity as wild type UVRB protein in participating in UVRAB-mediated anthramycin release. Both N51A and UVRB* retain ATPase activity, although in the presence of UVRA and UVRC proteins the former has incision activity, whereas the latter shows decreased incision activity.

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