# Inhibition of Rad3 DNA Helicase Activity by DNA Adducts and Abasic Sites: Implications for the Role of a DNA Helicase in Damage-Specific Incision of DNA†

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ABSTRACT: The yeast nucleotide excision repair gene *RAD3* is absolutely required for damage-specific incision of DNA. Rad3 protein is a DNA helicase, and previous studies have shown that its catalytic activity is inhibited by ultraviolet (UV) radiation damage. This inhibition is observed when base damage is confined to the DNA strand on which Rad3 protein binds and translocates, and not when damage is present exclusively on the complementary strand. In the present study, we show that Rad3 DNA helicase activity is inhibited in an identical strand-specific fashion by bulky base adducts formed by treating DNA with the antineoplastic agent cisplatin or the antibiotic compound CC-1065, which alter the secondary structure of DNA in different ways. In addition, Rad3 helicase activity is inhibited by small adducts generated by treatment of DNA with diethyl sulfate and by the presence of sites at which pyrimidines have been lost (abasic sites). No inhibition of Rad3 helicase activity was detected when DNA was methylated at various base positions. Cisplatin-modified single-stranded DNA and poly(deoxyuridylic acid) containing abasic sites are more effective competitors for Rad3 helicase activity than their undamaged counterparts, suggesting that Rad3 protein is sequestered at such lesions, resulting in the formation of stable Rad3 protein–DNA complexes. The observations of strand-specific inhibition of Rad3 helicase activity and the formation of stable complexes with the covalently modified strand suggest a general mechanism by which the *RAD3* gene product may be involved in nucleotide excision repair in yeast.

DNA is subject to damage by environmental agents or by endogenous cellular reactions, and the genetic integrity of all living organisms is protected by a complex array of DNA repair pathways (Friedberg, 1985). Nucleotide excision repair defines a DNA repair mode by which damaged bases are excised from the genome as components of single-stranded oligonucleotide fragments (Van Houten, 1990; Wood & Coverly, 1991), which may be as large as ~29 nucleotides in mammalian cells (Huang et al., 1992). Primary events associated with this repair mode include the specific recognition of sites of base damage in the genome and selective endonucleolytic cleavage of the damaged strand at these sites (Sancar & Sancar, 1988; Van Houten, 1990; Grossman & Yeung, 1990). The consequences of a defect in nucleotide excision repair are illustrated by the human DNA repair disorders xeroderma pigmentosum (XP) and Cockayne syndrome, which mainfest with hypersensitivity to the ultraviolet (UV) component of sunlight and, in the case of XP, with a high incidence of cancers of the exposed areas of the skin (Friedberg, 1985; Johnson & Squires, 1992).

Nucleotide excision repair recognizes and processes a remarkable diversity of DNA lesions induced by both physical and chemical agents. In vivo studies in yeast and mammalian cells indicate that substrates include highly distortive and helix-destabilizing modifications generated by UV radiation (Friedberg, 1988) or the chemotherapeutic agent *cis*-diamminedichloroplatinum(II) (cisplatin) (Wilborn & Brendel, 1989; Hannan et al., 1989), as well as modifications that stabilize the double helix, such as those produced by the antibiotic compounds anthramycin and CC-1065 (Petrusek et al., 1982; Jacobson et al., 1986). Recent studies in *Escherichia coli* indicate that in addition to bulky DNA adducts, nucleotide excision may also be involved in the repair of more subtle DNA modifications which do not result in gross perturbations of secondary structure, such as those produced by simple alkylating agents (Van Houten & Sancar, 1987; Voigt et al., 1989), and even sites of base loss (abasic sites) (Lin & Sancar, 1989). The molecular mechanisms whereby these chemically and structurally diverse DNA lesions are precisely located in the genome and discriminated as sites for damage-specific incision are the subject of intense scrutiny (Koo et al., 1991; Van Houten, 1990; Selby & Sancar, 1990; Grossman & Yeung, 1990), but are poorly understood in eukaryotes.

In the yeast *Saccharomyces cerevisiae*, *RAD3* is 1 of at least 10 nucleotide excision repair genes that have been identified by phenotypic complementation of UV-sensitive mutants (Friedberg, 1988, 1991a) or by homology with cloned human excision repair genes (Weeda et al., 1990). Detailed genetic analysis assigned *RAD3* to an early step in the nucleotide excision repair pathway (Cox & Game, 1974), and characterization of *rad3* mutants indicated that this gene is one of six genes that are absolutely required for DNA incision (Reynolds & Friedberg, 1981; Wilcox & Prakash, 1981). Subsequently, *RAD3* was shown to be homologous to the human nucleotide excision repair gene *ERCC2* (Weber et al., 1990) which specifically corrects the UV radiation sensitivity in XP cells from genetic complementation group D (Flejter et al., 1992).

Purification and biochemical characterization of the *RAD3* gene product have demonstrated that it is a single-stranded

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DNA-dependent ATPase that functions as a DNA helicase on partially duplex DNA substrates (Sung et al., 1987; Harosh et al., 1989; Matson & Kaiser-Rogers, 1990; Lohman, 1992). We recently reported that the Rad3 helicase activity is strand-specifically-inhibited by UV radiation damage (Naegeli et al., 1992a). Rad3 protein is sensitive to such damage in the DNA strand to which it binds and on which it presumably translocates during the unwinding reaction, but the enzyme is not inhibited by UV radiation damage located on the opposite strand (Naegeli et al., 1992a). We also directly demonstrated that Rad3 protein forms stable complexes with UV-irradiated DNA, presumably at or near sites of base damage, and in competition experiments incubation with UV-irradiated single-stranded DNA results in sequestration of the enzyme on the DNA (Naegeli et al., 1992a).

In the present studies, we show that inhibition of Rad3 helicase activity and sequestration of Rad3 protein on damaged single-stranded DNA are apparently quite general. Inhibition of Rad3 DNA helicase activity was observed regardless of whether modification of the DNA is helix-distortive (treatment with cisplatin), helix-stabilizing (treatment with CC-1065), or neither (sites of base loss). Inhibition of Rad3 helicase activity was also observed when the DNA substrate was covalently modified by the alkylating agent diethyl sulfate, suggesting that Rad3 protein can also recognize small chemical modifications of the DNA. Collectively, these observations suggest a mechanism by which Rad3 protein may be involved in searching for and precisely locating DNA damage in the genome of yeast cells.

## EXPERIMENTAL PROCEDURES

*Materials.* M13mp18 single-stranded DNA, T4 polynucleotide kinase, *dam* and *SssI* methylases, $N^6$-methyladenine-free λ DNA, and the restriction enzymes *Dnp*II and *Sal*I were purchased from New England Biolabs. Sephadex G-50 and all synthetic homopolymers were obtained from Pharmacia LKB. Uracil–DNA glycosylase of *E. coli* and the restriction enzymes *Bam*HI, *Eco*RI, and *Hin*dIII were obtained from Gibco BRL. [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol), [$\gamma$-$^{32}$P]ATP (6000 Ci/mmol), and [2,8-$^3$H]ATP (32 Ci/mmol) were from Amersham Corp. The compounds *cis*-diamminedichloroplatinum(II) (cisplatin), methyl methanesulfonate (MMS), and diethyl sulfate (DES) were from Sigma Chemical Co., and Bio-Gel A-5m was from Bio-Rad. Compound CC-1065 was generously provided by Dr. J. Patrick McGovren, The Upjohn Co., Kalamazoo, MI. Homogeneous preparations of Rad3 protein were obtained as previously described (Harosh et al., 1989; Naegeli et al., 1992a).

*Preparation of Partial Duplex Substrates for the DNA Helicase Assay.* A $^{32}$P-labeled 206-mer oligonucleotide was produced by single-stranded polymerase chain reaction and annealed to M13mp18 single-stranded DNA as described (Naegeli et al., 1992a). The 30-mer DNA primer (Harosh et al., 1989) was 5′ end-labeled by T4 polynucleotide kinase prior to being annealed to M13mp18 single-stranded DNA (Naegeli et al., 1992a). $(dA)_{25-30}$ and $(dT)_{25-30}$ were dephosphorylated and 5′ end-labeled as previously described (Naegeli et al., 1992b). For annealing, radiolabeled oligonucleotides were incubated with poly$(dT)_{167}$, poly$(dA)_{290}$, or poly$(dU)_{167}$ at an oligomer:polymer molar ratio of 1:1. Reactions included 10 mM Tris-HCl (pH 7.0), 100 mM NaCl, and 1 mM EDTA and were performed at room temperature. All partial duplex DNA substrates were purified through Bio-Gel A-5m gel filtration columns prior to incubation with Rad3 protein (Naegeli et al., 1992a).

*Preparation of Cisplatin-Modified DNA.* M13mp18 single-stranded DNA (300 μM in nucleotide equivalents) was incubated with cisplatin in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. The amount of cisplatin in the reaction is expressed as the molar ratio ($r$) of free cisplatin to nucleotide (Chu & Berg, 1987). After 20 h at 37 °C, reactions were stopped by the addition of NaCl to 0.5 M, and the DNA was ethanol-precipitated, washed, and resuspended in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, as described (Chu & Berg, 1987). The molar ratio ($r$) of free cisplatin to nucleotide determines the amount of cisplatin covalently bound to the DNA. On the basis of the results of previous studies using atomic absorption spectroscopy (Ushay et al., 1981), ~80% of the cisplatin is expected to be bound to DNA under the experimental conditions used in the present study. Hence, the ratios used in the experiments shown in Figure 1 ($r$ = 0.0024, $r$ = 0.012; and $r$ = 0.06) translate to 1 cisplatin adduct covalently bound per 520, 100, and 20 nucleotides respectively.

For the preparation of substrates used in the experiments shown in Figure 1B, M13mp18 single-stranded DNA (16 μM) or radiolabeled 206-mers (0.4 μM) were modified independently at molar ratios of free cisplatin to nucleotide of $r$ = 0.5 and $r$ = 10.0, respectively. After 20 h at 37 °C, the modified single-stranded DNA was purified through G-50 columns (Sambrook et al., 1989), followed by annealing to complementary DNA and purification of the resulting partial duplexes as described (Naegeli et al., 1992a). The *Bam*HI recognition sequence G′GATCC contains one GpG site which represents a potential target for cisplatin adducts. Hence, the extent of cisplatin modification was determined by the loss of sensitivity of duplex DNA to digestion by *Bam*HI (Chu & Berg, 1987). Following analysis of the *Bam*HI reaction products by denaturing polyacrylamide gel electrophoresis (Ogden & Adams, 1987), regions of the gel containing full-length 206-mers were visualized by autoradiography, excised, and assayed for Cerenkov radiation. This analysis revealed that 79% of the partial duplex molecules were protected from *Bam*HI digestion when the 206-mer was exposed to cisplatin at $r$ = 10 prior to being annealed whereas 68% of the fragments were protected when M13mp18 circular DNA was pretreated with cisplatin at $r$ = 0.5. Since the 206-mer oligonucleotide contains 15 sites with 2 or more adjacent guanine residues and the complementary region of M13mp18 contains 10 such sites, it can be estimated that a minimum of 11 and 6 of these sites, respectively, were modified (assuming that the *Bam*HI recognition sequence represents an average target site). At high $r$ values, adduct formation at ApG approaches the yield observed at GpG (Eastman, 1986). We obtained 64% and 51% protection from digestion from *Hin*dIII (recognition sequence, A′AGCTT) when the 206-mer oligonucleotide (containing 13 ApG sites) or the M13mp18 circle (containing 12 ApG sites) were modified, giving an estimate of at least 8 and 6 additional cisplatin adducts, respectively.

*CC-1065 Modification of Partial Duplex DNA.* Compound CC-1065 was dissolved in dimethyl sulfoxide to 270 μM and further diluted in aqueous solution immediately before use. Partial duplex DNA substrates (10 μM as nucleotide) were incubated in 10 mM Tris-HCl, pH 7.0, 100 mM NaCl, and 1 mM EDTA, in the presence of the indicated concentrations of CC-1065. After 2 h at room temperature, noncovalently bound CC-1065 was removed by purification through Bio-Gel A-5m columns (Naegeli et al., 1992a).

*Preparation of Alkylated DNA.* Partial duplex DNA substrates were incubated in 40 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol (DTT) for 18 h at 37 °C in the presence of the indicated concentrations of MMS or EMS. Following this treatment, the substrates were purified by gel filtration as described above. In order

to exclude the possibility that unreacted drug might contaminate the substrate preparations, a mock gel purification was performed on 50 μL of 1 M DES. Fractions corresponding to those containing partial duplex DNA substrates did not contain detectable activity that inhibited ATP hydrolysis or strand displacement by Rad3 protein.

For enzymatic methylation of DNA, partial duplex substrates were incubated at 37 °C with either *dam* or *SssI* methylase, using conditions recommended by the manufacturer. Following extraction with phenol/chloroform (1:1, pH 8.0), the DNA substrates were repurified by gel filtration as described above. The extent of enzymatic methylation was determined by digestion with *Dpn*II (*dam* methylase) or *Sal*I (*SssI* methylase) under conditions described by the manufacturer. In both cases, >90% protection of methylated substrates was observed, whereas control substrates were digested to completion.

*Preparation of Alkylated DNA for the ATPase Assay.* Phage λ DNA containing no $N^6$-methyladenine was reacted with MMS or DES as described (Lawley & Brookes, 1963), and the DNA was purified from unreacted compound (Rhodes, 1989). For enzymatic methylation, $N^6$-methyladenine-free λ DNA was incubated with *dam* or *SssI* methylase. Immediately prior to use in the ATPase assay, the double-stranded λ DNA was heat-denatured by incubation at 94 °C for 2 min followed by rapid chilling in ice water.

*Preparation of DNA Substrates Containing Abasic Sites.* Unlabeled or 5′ end-labeled (Sambrook et al., 1989) poly-$(dU)_{167}$ was adjusted to a concentration of 300 μM in 25 mM Hepes, pH 8.0, 25 mM KCl, 0.5 mM EDTA, and 0.5 mM DTT and incubated with uracil–DNA glycosylase (20 units/mL) for the indicated time periods. Incubations were stopped by the addition of 4 volumes of 10 mM sodium citrate buffer to a final pH of 5.0, the uracil–DNA glycosylase was denatured by heating the reactions to 70 °C for 10 min, and samples were stored at −20 °C.

*ATPase and DNA Helicase Assays.* ATPase and DNA helicase activities of Rad3 protein were determined and expressed as previously described (Harosh et al., 1989; Naegeli et al., 1992a,b).

## RESULTS

*Strand-Specific Inhibition of the DNA Helicase Activity of Rad3 Protein by Cisplatin Adducts.* The prevalent adduct formed by the interaction of cisplatin with DNA is an intrastrand cross-link between the $N^7$ atoms of adjacent guanine nucleotides (Sherman & Lippard, 1987). In order to determine the effect of cisplatin adducts on Rad3 DNA helicase activity, we modified M13 single-stranded DNA at different molar ratios of free cisplatin to nucleotide ($r$) ranging between $r$ = 0.0024 and $r$ = 0.06. The DNA was purified from unreacted cisplatin and annealed to a complementary $^{32}$P-labeled 206-mer oligonucleotide. We confirmed by denaturing polyacrylamide gel electrophoresis that cisplatin modification of single-stranded DNA prior to annealing to the complementary oligonucleotide precluded the formation of interstrand cross-links (data not shown). Partial inhibition of Rad3 DNA helicase activity (~22%) was observed when M13 single-stranded DNA was modified at a free cisplatin to nucleotide ratio of $r$ = 0.0024 (Figure 1A), which translates to the expected formation of 1 cisplatin adduct per 520 nucleotides (see Experimental Procedures). More complete inhibition of Rad3 helicase activity was observed when the DNA was modified at drug to nucleotide ratios of $r$ = 0.012 and $r$ = 0.06 (Figure 1A).

Previous studies using UV-irradiated partially duplex DNA demonstrated that Rad3 protein is uniquely sensitive to damage

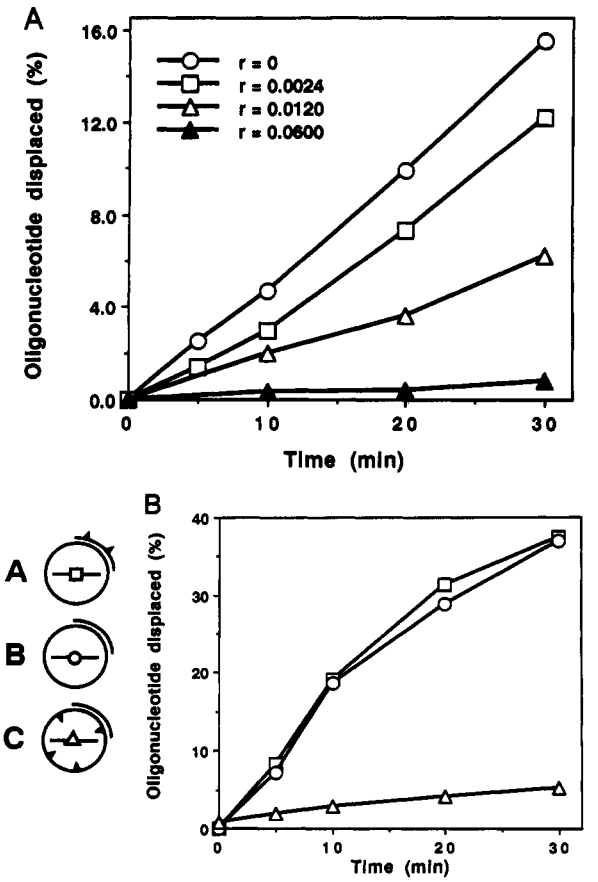


FIGURE 1: Inhibition of Rad3 helicase activity by cisplatin–DNA adducts. (A) Cisplatin dose dependence. Rad3 protein (40 ng) was incubated with a partial duplex DNA substrate (75 nM, in nucleotides) constructed by annealing a $^{32}$P-labeled 206-mer oligonucleotide to M13 single-stranded DNA. The M13 DNA was modified with cisplatin at the indicated ratios of drug to nucleotide ($r$). After the indicated reaction times, the percentage of oligonucleotides released was detected by polyacrylamide gel electrophoresis and quantified as described under Experimental Procedures (averages of duplicate determinations). (B) DNA strand specificity. Rad3 protein (80 ng) was incubated with 75 nM undamaged helicase substrate (substrate B) or with equimolar amounts of substrates carrying cisplatin adducts either in the 206-mer oligonucleotide (substrate A) or in the M13 single-stranded DNA (substrate C). Substrates A and C contained at least 19 and 12 adducts, respectively. After the indicated times, the percentage of oligonucleotides displaced was quantified as described under Experimental Procedures (mean values of three determinations). Squares, substrate A; circles, substrate B; triangles, substrate C.

located on the circular single strand on which the enzyme binds and translocates with 5′→3′ polarity during the unwinding reaction (Naegeli et al., 1992a). We generated a substrate containing a very high frequency of cisplatin adducts exclusively in the radiolabeled oligonucleotide (Figure 1B, substrate A) by modifying the fragment at a cisplatin to nucleotide ratio of $r$ = 10.0, followed by purification of the damaged fragment and annealing to single-stranded circular M13 DNA. The resulting partial duplex substrate carried a minimum of 19 cisplatin adducts per annealed oligonucleotides (see Experimental Procedures). Control substrates were constructed by annealing undamaged (Figure 1B, substrate B) or cisplatin-damaged M13 single-stranded DNA (Figure 1B, substrate C) to the undamaged 206-mer. The kinetics and extent of oligonucleotide displacement by Rad3 protein were indistinguishable with substrates A or B (Figure 1B), despite the fact that in substrate A the displaced oligonucleotide was extensively modified by cisplatin. On the other hand, unwinding of substrate C by Rad3 protein was completely inhibited (Figure 1B). These results confirmed that Rad3

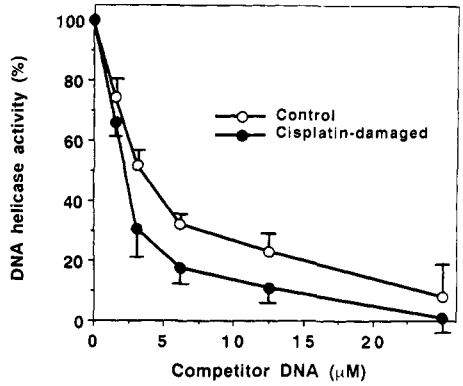


FIGURE 2: Sequestration of Rad3 protein on cisplatin-modified single-stranded DNA. Rad3 protein (20 ng) was incubated for 10 min with the indicated concentrations of competitor M13 single-stranded DNA. The M13 DNA was either undamaged (open circles) or cisplatin-damaged ($r \approx 0.06$, filled circles). Helicase substrate consisting of 30-mer DNA primers annealed to complementary single-stranded DNA was then added to the reactions at a concentration of 0.3 μM. After a further 30 min of incubation, samples were analyzed by gel electrophoresis, and the percentage of primers displaced was quantified as indicated under Experimental Procedures. Helicase activity (mean values of three determinations ± SD) is expressed as the percentage of activity observed in the absence of competitor (60.4% primers displaced).

DNA helicase activity is sensitive to base damage located uniquely in the strand on which the enzyme translocates.

*Sequestration of Rad3 Protein on Cisplatin-Damaged Single-Stranded DNA.* We previously reported that UV radiation damage in single-stranded DNA results in the formation of stable Rad3 protein–DNA complexes (Naegeli et al., 1992a). As a consequence, preincubation with UV-irradiated single-stranded DNA results in more pronounced competition for subsequent Rad3 DNA helicase activity than does preincubation with unirradiated single-stranded DNA (Naegeli et al., 1992a). In order to determine whether Rad3 protein was sequestered on DNA treated with cisplatin, we preincubated the enzyme for 10 min with varying amounts of undamaged or cisplatin-modified single-stranded DNA at a drug to nucleotide ratio of $r = 0.06$ (Figure 2). We then added a substrate for DNA helicase activity consisting of circular single-stranded DNA annealed to a complementary 5′ end-labeled 30-mer oligonucleotide. Following incubation for a further 30 min, the cisplatin-treated single-stranded DNA was found to be a more potent competitor than undamaged single-stranded DNA at all concentrations tested (Figure 2). For example, when 3 μM undamaged M13 DNA was used as the competitor, helicase activity was reduced to 51.7% of that measured in its absence (Figure 2). On the other hand, 3 μM M13 DNA treated with cisplatin reduced the helicase activity to 28.9% of the control reaction (Figure 2). A quantitatively comparable result was previously observed with UV-irradiated competitor DNA (Naegeli et al., 1992a). This was directly shown to result from a >20-fold increase in the half-life of Rad3–DNA complexes (Naegeli et al., 1992a).

*Strand-Specific Inhibition of Rad3 DNA Helicase Activity by CC-1065 Adducts.* The compound CC-1065 is a potent antibiotic that reacts at the $N^3$ position of adenine, forming bulky DNA adducts (Reynolds et al., 1986). This compound exclusively modifies and specifically recognizes the sequence 5′(A/T)(A/T)A*, where an asterisk indicates the covalently modified adenine residue (Reynolds et al., 1986; Hurley et al., 1984; Lee et al., 1991). In contrast to the helix-destabilizing and helix-distortive modifications of DNA caused by UV radiation or cisplatin (Husain et al., 1988; Rice et al., 1988), CC-1065 increases the melting point of double-stranded DNA dramatically and also decreases its sensitivity to S1 nuclease (Reynolds et al., 1986). The partial duplex DNA substrate obtained by annealing a $^{32}P$-labeled 206-mer oligonucleotide to M13 single-stranded DNA contains five potential 5′(A/T)(A/T)A* binding sites for CC-1065 on each strand of the duplex region. Modification of the partially duplex DNA at nanomolar concentrations of CC-1065 resulted in inhibition of Rad3 DNA helicase activity (Figure 3).

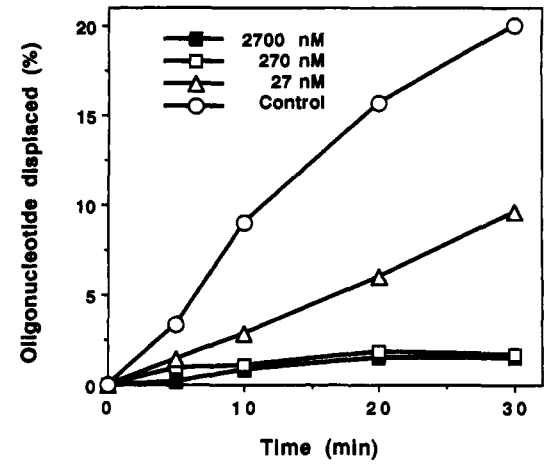


FIGURE 3: Inhibition of Rad3 DNA helicase activity by CC-1065–DNA adducts. Rad3 protein (50 ng) was incubated with partially duplex DNA substrates (75 nM) constructed by annealing radiolabeled 206-mers to complementary M13 single-stranded DNA. The substrates were undamaged (open circles) or damaged with the indicated concentrations of compound CC-1065, ranging between 27 nM and 2.7 μM (averages of duplicate determinations).

In the experiments just described, both strands of the duplex region contained sites for modification by CC-1065. To explore the question of strand specificity, we constructed partially duplex deoxyribopolymer substrates in which covalent modification of adenine bases was confined to one polynucleotide chain. End-labeled $(dT)_{25-30}$ was annealed to poly$(dA)_{290}$, or end-labeled $(dA)_{25-30}$ was annealed to poly$(dT)_{167}$. Following incubation of these partially duplex molecules with CC-1065 and their purification by gel filtration, we demonstrated that covalent modification of both poly(dA)·oligo(dT) and poly(dT)·oligo(dA) dramatically increased their thermal stability. $(dT)_{25-30}$ was readily displaced from undamaged poly$(dA)_{290}$ when the duplex molecules were incubated at 90 °C for 5 min in the absence of any Rad3 protein (Figure 4A, lanes 4 and 5). In contrast, substrates modified in the presence of 270 nM or 2.7 μM CC-1065 remained stable when heated at 90 °C (Figure 4A, lanes 6–9). Modification of poly(dT)·oligo(dA) increased its thermal stability to the same extent (compare panels A and B of Figure 4).

Consistent with the presence of base damage in $(dA)_{25-30}$, this polymer migrated in denaturing gels more slowly than undamaged $(dA)_{25-30}$, and also manifested greater electrophoretic heterogeneity (Figure 4C, lanes 1–3). Additionally, as is commonly observed with $N^3$-alkylated species (Reynolds et al., 1986), incubation of covalently modified $(dA)_{25-30}$ at 90 °C for 60 min at neutral pH resulted in cleavage of the molecules (Figure 4C, lanes 5–8), whereas native $(dA)_{25-30}$ was resistant to this treatment (Figure 4C, lane 4). $(dT)_{25-30}$ incubated with CC-1065 exhibited the same electrophoretic properties as untreated material, confirming that covalent modification by CC-1065 was specifically targeted to poly(dA) molecules (data not shown). Analysis of the modified substrates by denaturing gel electrophoresis in the presence of formamide demonstrated the absence of interstrand cross-links (data not shown). These results indicate that we generated partial duplex polymer substrates in which exclu-

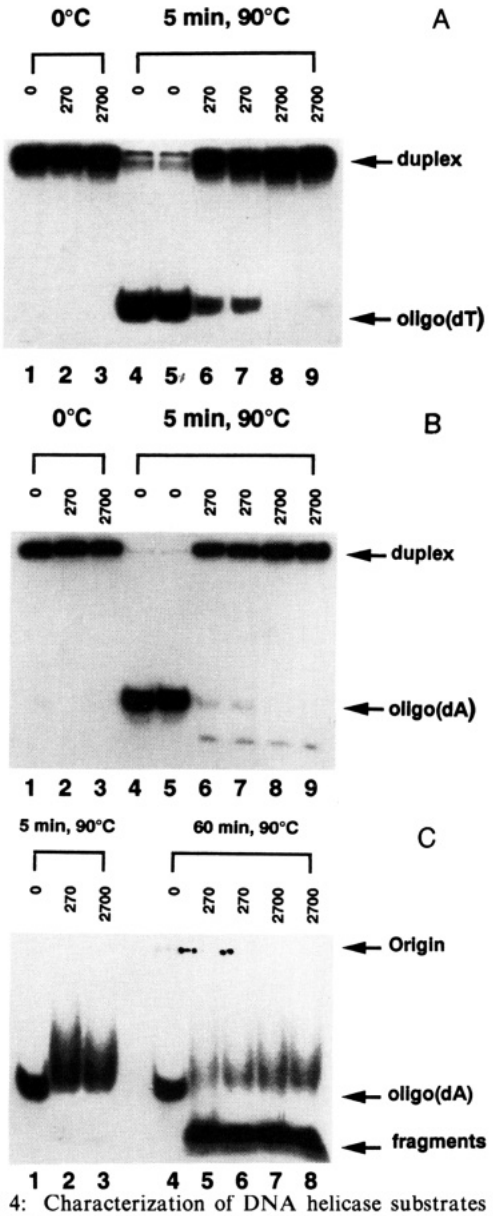


FIGURE 4: Characterization of DNA helicase substrates strand-specifically-modified by CC-1065. (A) Analysis of poly(dA)·oligo(dT) on a nondenaturing 10% polyacrylamide gel. The partial duplex was modified with the indicated concentrations of CC-1065 (0–2700 nM) and purified by gel filtration as indicated under Experimental Procedures. The substrates were stored on ice (lanes 1–3) or incubated for 5 min at 90 °C (lanes 4–9, in duplicate). The positions of partially duplex substrates and of released 5′ end-labeled $(dT)_{25-30}$ are indicated by the arrows. (B) Analysis of poly(dT)·oligo(dA) on a nondenaturing 10% polyacrylamide gel. The substrates were modified with CC-1065, purified, and stored on ice (lanes 1–3) or incubated for 5 min at 90 °C (lanes 4–9, in duplicate). The small bands in lanes 6–9 represent a minor fraction of covalently modified $(dA)_{25-30}$ that was degraded during the incubation at 90 °C. (C) Analysis of poly(dT)·oligo(dA) on a denaturing 10% polyacrylamide gel (Ogden & Adams, 1987). After CC-1065 modification and purification, the substrates were stored on ice (lanes 1–3) or incubated for 60 min at 90 °C (lanes 4–8, the modified samples are shown in duplicate). Prior to electrophoresis, all samples were heated for 5 min at 90 °C in the presence of 50% (v/v) formamide. The positions of the gel origin, of native 5′ end-labeled $(dA)_{25-30}$, and of the thermal cleavage products are indicated by the arrows.

sively either the DNA strand on which Rad3 protein binds and translocates (Figure 4A) or the opposite strand (Figure 4B,C) was modified by the helix-stabilizing agent CC-1065.

In poly(dA)·oligo(dT) modified by CC-1065 at a concentration of 270 nM, the presence of DNA adducts in the poly(dA) strand inhibited Rad3 helicase activity by ~95% (Figure 5). Since CC-1065 modifies poly(dA) only when the polymer

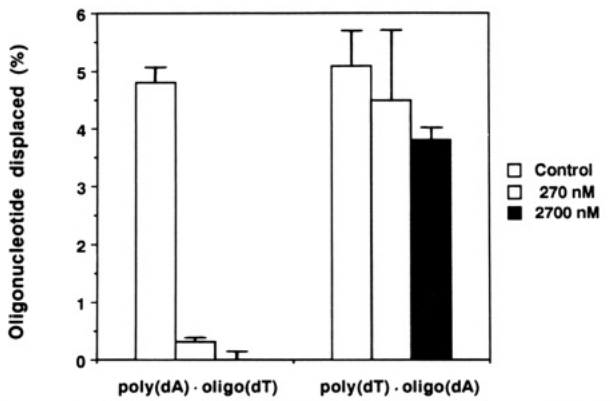


FIGURE 5: Strand-specific inhibition of Rad3 helicase activity by CC-1065. Rad3 protein (40 ng) was incubated with the indicated partially duplex DNA substrates (150 nM; 40 000 dpm per reaction). The substrates were either undamaged ("Control") or modified with 270 or 2700 nM CC-1065. After 30 min, samples were analyzed by polyacrylamide gel electrophoresis, and the percentages of oligonucleotides displaced (mean values of four experiments ± SD) were quantified as described under Experimental Procedures.

is annealed to complementary oligo(dT) (Reynolds et al., 1986), this result demonstrates that modification of single-stranded regions of the substrate is not required for inhibition of Rad3 helicase activity. DNA helicase activity was not significantly affected by the presence of CC-1065 adducts in the opposite strand, as indicated by the efficient unwinding of poly(dT)·oligo(dA) substrates modified by the same concentration of the compound (Figure 5). Similarly, modification by CC-1065 at a concentration of 2.7 $\mu$M completely blocked the unwinding of poly(dA)·oligo(dT), but exerted a minimal effect on the unwinding of poly(dT)·oligo(dA) (Figure 5). Consistent with the electrophoretic characterization of damaged $(dA)_{25-30}$ shown in Figure 4C, $(dA)_{25-30}$ molecules displaced by Rad3 protein from modified poly(dT)·oligo(dA) exhibited reduced electrophoretic mobility compared to undamaged $(dA)_{25-30}$ and migrated as a broad band (data not shown).

*Effect of DNA Methylation on Rad3 Enzyme Activity.* To determine the effect of smaller adducts on the Rad3 DNA helicase activity, partial duplex DNA helicase substrates were reacted with the alkylating agent MMS under experimental conditions designed to modify ~1% of bases in the DNA substrate (Lawley & Brookes, 1963), i.e., ~70 bases per partial DNA duplex molecule. MMS methylates primarily the $N^7$ of guanine and the $N^3$ of adenine, with relative yields of ~85% and ~10%, respectively (Singer & Grunberger, 1983). The presence of these methylations in partially duplex DNA did not inhibit the displacement of oligonucleotides by Rad3 protein (Figure 6). Additionally, MMS modification and subsequent denaturation of λ DNA did not reduce its ability to activate hydrolysis of ATP by Rad3 protein (data not shown).

The observation that methylation targeted to particular base positions did not inhibit Rad3 helicase and ATPase activities was extended by using bacterial methylases. Incubation of partially duplex DNA with *E. coli dam* methylase (Smith & Kelley, 1984) resulted in the formation of $N^6$-methyladenine at two GATC sites in the duplex region, and the *SssI* methylase of *Spiroplasma* sp. strain MQ1 (Renbaum et al., 1990) produced 5-methylcytosine at 17 CG positions (see Experimental Procedures for details). In neither case did the methyl groups inhibit Rad3 DNA helicase activity (Figure 6). Similarly, modification of phage λ DNA by these enzymes did not reduce its effectiveness as an activator in the Rad3 ATPase assay after denaturation of the DNA (data not

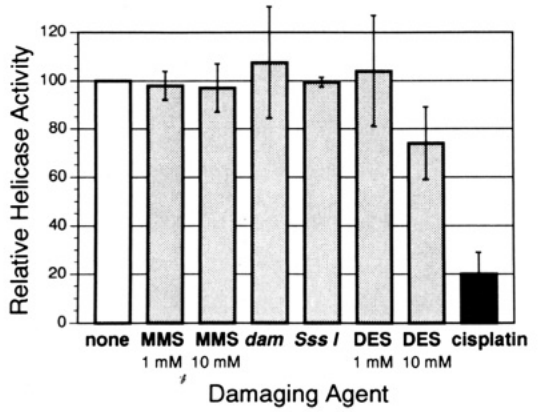


FIGURE 6: Effect of various DNA alkylations on Rad3 helicase activity. Partially duplex DNA substrates consisting of $^{32}$P-labeled 206-mers annealed to M13 single-stranded DNA were incubated with *dam* or *SssI* methylases, or reacted with MMS or DES as designed under Experimental Procedures. For comparison, the DNA substrate was also modified with cisplatin ($r = 0.03$). The purified substrates were incubated with Rad3 protein (50 ng) at a concentration of 76 nM. After 30 min, the fraction of 206-mers displaced (mean values of 3–5 experiments ± SD) was quantified and is expressed as the percentage of oligonucleotide displaced in the control reaction with undamaged DNA (24.5% oligonucleotide displaced).

shown). Collectively, these results suggest that the interaction of Rad3 protein with DNA is insensitive to methylation at the $N^7$ of guanine, the $N^3$ or $N^6$ of adenine, and the $C^5$ of cytosine.

*Inhibition of Rad3 Enzyme Activity by DNA Ethylation.* The partial duplex DNA helicase substrate was also modified with the ethylating agent DES. Under identical experimental conditions, DES introduces larger alkyl groups at the same positions in adenine and guanine that are targeted by MMS, but at an ~10-fold lower frequency at saturating concentrations of alkylating agent (Singer & Grunberger, 1983). Hence, incubation of the partial duplex DNA substrate with 10 mM DES is likely to result in the alkylation of ~1/1000 bases, or ~7 alkylations per partial duplex molecule, whereas treatment with 1 mM DES is expected to yield <1 alkylation per duplex molecule. As shown in Figure 6, treatment with 10 mM DES caused a substantial (23%) reduction in oligonucleotide displacement by Rad3 protein. The failure to detect inhibition of Rad3 helicase activity with 1 mM DES (Figure 6) is consistent with the expected level of alkylation of bases at that concentration (Singer & Grunberger, 1983). In parallel, modification of phage λ DNA with 10 mM DES and subsequent denaturation of the DNA significantly reduced its ability to activate Rad3 ATP hydrolysis (data not shown).

The extent of the inhibition of Rad3 helicase activity in the presence of ethylated DNA (~23%) (Figure 6) is similar to that observed when DNA was UV-irradiated at a dose of 150 J/m$^2$ (Naegeli et al., 1992a), which generates ~1 cyclobutane pyrimidine dimer per 1200 bases (Bardwell and Friedberg, unpublished observations), and other photoproducts at a lower level (Friedberg, 1985). This value is in good agreement with the expected levels of ethylated adenine and guanine (~1/1000 bases) and suggests that the observed inhibition cannot be attributed to minor alkylation products such as ethyltriesters or apurinic sites, since these modifications are expected to occur at a frequency of <1 per 6000 nucleotides (Singer & Grunberger, 1983).

*Inhibition of Rad3 ATPase and Helicase Activities by Abasic Sites in DNA.* Abasic sites in DNA can be generated by spontaneous hydrolytic cleavage of purine nucleotides (Lindahl & Nyberg, 1972) and by the action of different DNA glycosylases. In order to explore the effect of sites of base loss on the Rad3 ATPase and helicase activities, we utilized substrates in which sites of base loss could be generated in a controlled manner by enzyme-catalyzed removal of uracil from poly(dU). Unlabeled or 5′ end-labeled poly(dU)$_{167}$ was incubated with uracil–DNA glycosylase of *E. coli* (Lindahl et al., 1977) for increasing periods of time. When analyzed on polyacrylamide gels, the enzyme-treated poly(dU)$_{167}$ migrated progressively faster than the intact species (Figure 7A, lanes 1–5), consistent with the increasing accumulation of abasic sites. After 50 min of incubation, the substrate migrated as a broad band in both 4% (Figure 7A, lane 5) and 20% (data not shown) polyacrylamide gels. In contrast, the electrophoretic mobility of poly(dU)$_{167}$ incubated in the absence of the DNA glycosylase remained indistinguishable from that of control poly(dU)$_{167}$ (data not shown).

The electrophoretic mobility of poly(dU)$_{167}$ molecules containing abasic sites was unaffected by subsequent incubation under Rad3 protein reaction conditions (data not shown), consistent with earlier studies showing that sites of base loss in polydeoxyribonucleotides are quite stable at physiological pH and temperature (Lindahl & Andersson, 1972). On the other hand, poly(dU)$_{167}$ incubated with uracil–DNA glycosylase was clearly alkali-sensitive (Figure 7A, lanes 7–10). When the alkaline hydrolysis reaction products were resolved on 20% polyacrylamide gels, untreated poly(dU)$_{167}$ remained near the origin of the gel (Figure 7B, lane 1), whereas preincubation with uracil–DNA glycosylase produced a ladder of alkaline cleavage products (Figure 7B, lanes 2–8), indicative of the progressive accumulation of abasic sites. Preincubation of poly(dU)$_{167}$ with uracil–DNA glycosylase for 2 min was sufficient to generate at least one abasic site per molecule of polynucleotide (Figure 7B, lane 2).

The ability of poly(dU)$_{167}$ containing sites of base loss to activate ATP hydrolysis by Rad3 protein was progressively reduced as a function of preincubation with DNA glycosylase (Figure 7C). This effect was not observed when poly(dT)$_{167}$ was preincubated with uracil–DNA glycosylase (Figure 7C), or when poly(dU)$_{167}$ was preincubated in the absence of the glycosylase (Figure 7C). These experiments indicate that an abasic deoxyribose–phosphate backbone is unable to induce the conformational changes in Rad3 protein that are presumably required for ATP binding and/or hydrolysis.

To directly examine the effect of abasic sites on Rad3 helicase activity, we annealed 5′ end-labeled (dA)$_{25-30}$ molecules to undamaged poly(dU)$_{167}$, or to poly(dU)$_{167}$ previously incubated with uracil–DNA glycosylase. The displacement of (dA)$_{25-30}$ by Rad3 protein was reduced as a function of the number of abasic sites in the poly(dU)$_{167}$ strand (Table I).

*Sequestration of Rad3 Protein on Poly(dU)$_{167}$ Containing Abasic Sites.* The observation that Rad3 helicase activity is inhibited both by chemical modification and by sites of base loss prompted us to investigate how Rad3 protein might interact with abasic sites. As was previously observed with UV-irradiated (Naegeli et al., 1992a) or cisplatin-damaged DNA (see above), we found that poly(dU)$_{167}$ containing abasic sites was a more effective competitor for Rad3 helicase activity than was undamaged poly(dU)$_{167}$. When tested at a concentration of 0.75 μM (in phosphate equivalent), undamaged poly(dU)$_{167}$ reduced subsequent Rad3 helicase activity by 43.5%, whereas poly(dU)$_{167}$ preincubated with uracil–DNA glycosylase for 5 or 10 min reduced Rad3 helicase activity by 60.5% and 65.8%, respectively (mean values of three independent experiments; data not shown). Thus, an additional ~20% of active Rad3 enzyme molecules were apparently sequestered on the damaged competitor, and were not available for unwinding of the helicase substrate. Similar results were obtained at all poly(dU)$_{167}$ concentrations tested (data not

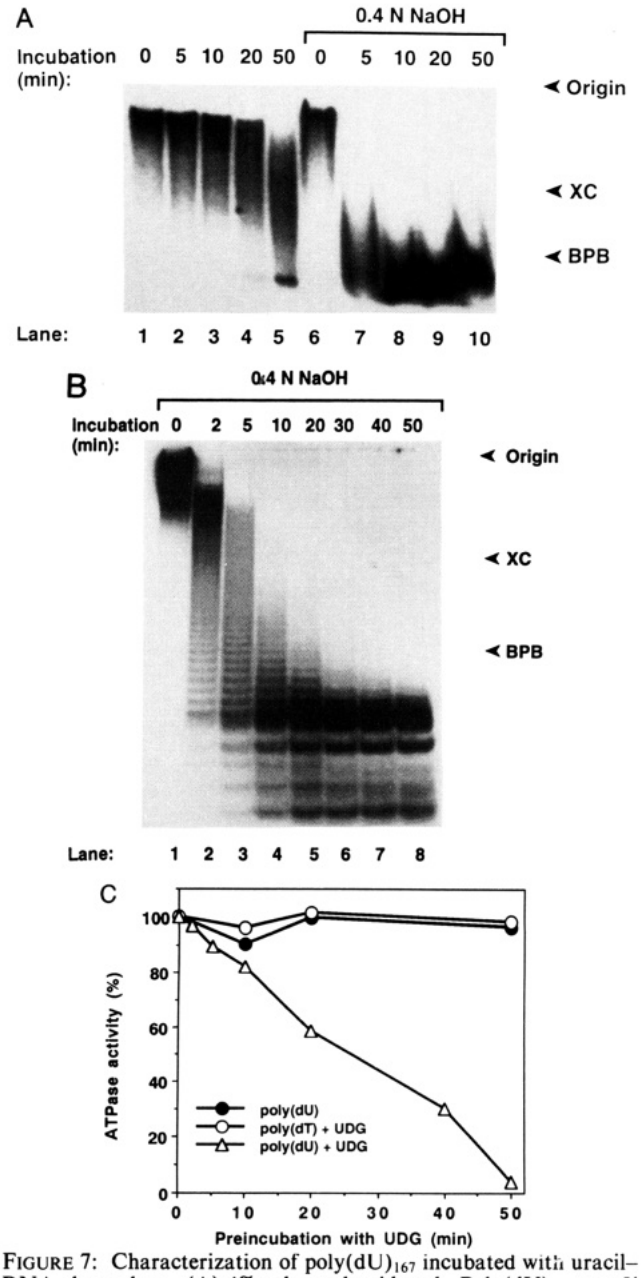


FIGURE 7: Characterization of poly(dU)$_{167}$ incubated with uracil–DNA glycosylase. (A) 4% polyacrylamide gel. Poly(dU)$_{167}$ was 5′ end-labeled and incubated with uracil–DNA glycosylase for the indicated times (minutes) as described under Experimental Procedures. Samples were directly loaded onto the gel (lanes 1–5) or preincubated for 15 min at 37 °C in the presence of 0.4 N NaOH (lanes 6–10). The positions of the gel origin and of the dye markers xylene cyanole and bromphenol blue are indicated by the arrows. (B) Analysis of the alkaline hydrolysis reaction products on a 20% polyacrylamide gel. After preincubation with uracil–DNA glycosylase for the indicated times (in minutes), $^{32}$P-labeled poly(dU)$_{167}$ was incubated with 0.4 N NaOH for 15 min at 37 °C, and the reaction products were resolved by gel electrophoresis. (C) Poly(dU)$_{167}$ was preincubated for the indicated times (minutes) with uracil–DNA glycosylase (UDG). ATPase reactions contained 20 ng of Rad3 protein and 6 $\mu$M (in phosphate equivalents) of the various poly(dU)$_{167}$ derivatives. After 30 min, ATP hydrolysis (averages of duplicate determinations) was measured by thin-layer chromatography and expressed as the percentage of activity obtained in the presence of undamaged poly(dU)$_{167}$ (triangles). Control ATPase reactions contained equal concentrations of poly(dT)$_{167}$ preincubated with the glycosylase (open circles) or poly(dU)$_{167}$ that was preincubated in the absence of the glycosylase (filled circles).

shown). Preincubation of the polymer with DNA glycosylase for periods longer than 10 min did not further increase its ability to compete for Rad3 helicase activity. On the contrary, extended preincubation progressively reduced competing ability (data not shown), suggesting that Rad3 protein does not interact with the deoxyribose–phosphate backbone after extensive base loss.

Table I: Inhibition of Rad3 Helicase Activity by Abasic Sites in Poly(dU)·Oligo(dA)[a]

| incubn of poly(dU)$_{167}$ with UDG prior to annealing (min) | (dA)$_{25-30}$ displaced by Rad3 protein (%) |
|---|---|
| 0 | 38.1 ± 5.4 |
| 2 | 26.0 ± 3.6 |
| 5 | 19.9 ± 2.4 |

[a] Poly(dU)$_{167}$ was preincubated with uracil–DNA glycosylase as described under Experimental Procedures and annealed to 5′ end-labeled (dA)$_{25-30}$ for 15 min at room temperature in 10 mM Tris-HCl buffer, pH 7.0, containing 100 mM NaCl and 1 mM EDTA. After purification by gel filtration, the substrates (38 nM, in nucleotide equivalents) were immediately incubated with Rad3 protein (80 ng) and ATP (1 mM). After 30 min, displaced oligonucleotides were detected by gel electrophoresis and quantified (mean values of three experiments ± SD). All values were corrected for background by subtracting the release of oligonucleotide (<5%) in control incubations without ATP.

## DISCUSSION

We have previously shown that the DNA helicase activity of Rad3 protein is inhibited by UV radiation-induced damage in partially duplex DNA (Naegeli et al., 1992a). Additionally, Rad3 protein discriminates between DNA and RNA in partially duplex substrates (Naegeli et al., 1992b). In both cases, the ability of the protein to act as a helicase is specifically inhibited only when the polynucleotide strand to which it binds and translocates is structurally altered. We also demonstrated that UV radiation damage induces the formation of stable Rad3 protein–DNA complexes, presumably at or near sites of base damage (Naegeli et al., 1992a). We have determined that the half-life of Rad3 protein–DNA complexes increased from 3.9 min in the absence of UV radiation damage to 84.0 min in the presence of UV radiation damage (Naegeli et al., 1992a). As a consequence, preincubation with UV-irradiated single-stranded DNA results in a more pronounced competition for subsequent Rad3 DNA helicase activity than does preincubation with unirradiated single-stranded DNA (Naegeli et al., 1992a).

In the present study, we have investigated the generality of these phenomena by examining a spectrum of chemical alterations in DNA. Our results demonstrate that covalent modification of DNA by either cisplatin or compound CC-1065, which produce adducts of very different chemical composition and affect the secondary structure of DNA in diametrically opposite ways, also inhibits Rad3 helicase activity in a strand-specific manner. Additionally, inhibition of Rad3 helicase activity was observed when the DNA substrate was perturbed by ethylation or by the loss of bases. In the presence of cisplatin adducts or sites of base ethylation, we observed a substantial (~25%) inhibition of Rad3 helicase activity at a frequency of only 1 lesion per 500–1000 bases of partially duplex DNA, regardless of the type of DNA modification tested. These values are in good agreement with the extent of inhibition (20–30%) observed when partially duplex DNA was UV-irradiated at a dose of 150 J/m$^2$ (Naegeli et al., 1992a; H. Naegeli, L. Bardwell, and E. C. Friedberg, unpublished observations), which generates ~1 cyclobutane pyrimidine dimer per 1200 bases (Bardwell and Friedberg, unpublished observations), and other photoproducts at a lower level (Friedberg, 1985).

The strand specificity for inhibition of Rad3 helicase activity by DNA ethylation and base loss was not directly investigated. However, this specificity may be reasonably inferred from

the observation that chemically and structurally unrelated DNA lesions such as UV radiation damage (Naegeli et al., 1992a), cisplatin damage, and CC-1065 adducts (this study) exhibit the common property of strand-specifically-inhibiting Rad3 DNA helicase activity. Hence, it is likely that all covalent DNA modifications that inhibit the DNA helicase activity of Rad3 protein do so in a strand-specific manner.

As is the case for inhibition of Rad3 helicase activity, it appears that sequestration of Rad3 protein at or near sites of base damage is a general phenomenon. In the present study, we have shown that cisplatin-modified single-stranded DNA and poly(deoxyuridylic acid) containing abasic sites are more effective competitors for Rad3 helicase activity than undamaged single-stranded DNA or poly(deoxyuridylic acid). Collectively, these and earlier studies (Naegeli et al., 1992a) indicate that DNA modifications as diverse as UV radiation damage, cisplatin adducts, and the loss of bases result in the sequestration and stable binding of Rad3 protein on DNA.

Genetic studies indicate that the nucleotide excision repair pathway of *S. cerevisiae* is involved in the repair of simple alkylations of DNA, including DNA methylation (Cooper & Waters, 1987; Hoekstra & Malone, 1986). However, the cellular response in yeast to simple DNA alkylations appears to be complex and is likely to involve additional DNA repair systems (Cooper & Waters, 1987). In the present studies, the covalent addition of methyl groups at $N^7$ guanine, $N^3$ adenine, $N^6$ adenine, or $C^5$ cytosine had no detectable effect on the DNA helicase activity of Rad3 protein. These findings suggest a size threshold with respect to the ability of base adducts at the above-mentioned positions to inhibit Rad3 helicase activity. In fact, we observed that CC-1065 adducts at $N^3$ adenine inhibited Rad3 helicase activity, whereas the presence of $N^3$-methyladenine had no effect. Similarly, inhibition was observed when DNA was treated with DES, which ethylates DNA predominantly at $N^7$ guanine (Singer & Grunberger, 1983), or with cisplatin, which generates bulky DNA adducts at the $N^7$ position (Sherman & Lippard, 1987). In contrast, $N^7$-methylguanine adducts present in the DNA at a frequency ~10-fold higher than $N^7$-ethylguanine adducts were not inhibitory.

Cisplatin and CC-1065 adducts are belived to result in distinct alterations in the secondary structure of duplex DNA. Cisplatin adducts induce localized unwinding of the DNA duplex with bending of the DNA helix by as much as 40° in the direction of the major groove (Rice et al., 1988). In contrast, CC-1065 adducts dramatically stabilizes double-stranded DNA without causing gross alterations in the secondary structure (Reynolds et al., 1986). Recent studies indicate that CC-1065 induces slight bending of the DNA into the minor groove (Lee et al., 1991), with associated narrowing of the groove. This conformational perturbation is believed to be similar to that produced spontaneously by adenine tracts five to six bases in length (Hagerman, 1990). Thus, inhibition of Rad3 helicase activity is apparently independent of the particular conformation adopted by DNA in the presence of base damage. This view is supported by the observation that Rad3 helicase activity is also inhibited by both apurinic and apyrimidinic sites in DNA, neither of which are believed to result in significant alterations in the secondary structure of classical B-form DNA (Doetsch & Cunningham, 1990).

Another interesting property of compound CC-1065 is its strict requirement for double-stranded DNA, as it has been demonstrated that heat-denatured calf thymus DNA or poly(deoxyadenylic acid) is not modified by this agent (Reynolds et al., 1986). Hence, CC-1065 modification of the partially duplex DNA used in these studies is presumably confined to the 206 base pair double-stranded region. With the possible exception of a few short hairpin structures (Rasched & Oberer, 1986), the single-stranded portion of the substrate is not a target for CC-1065 modification. Similarly, in poly(dA)$_{290}$·(dT)$_{25-30}$, only adenine residues located within the duplex region are targeted by CC-1065. Since treatment of both substrates by CC-1065 results in marked inhibition of Rad3 DNA helicase activity, it is likely that modification of the double-stranded region of partially duplex DNA is sufficient for this inhibition. Hence, it is probable that in the case of cisplatin adducts, DNA ethylation, and sites of base loss, which do not discriminate between single-stranded and double-stranded DNA, base lesions positioned in the single-stranded portion of the helicase substrates are not necessary for the observed inhibition of Rad3 helicase activity, although these lesions may well contribute to the overall extent of enzyme inhibition.

It has been previously reported that several other DNA helicases are inhibited by DNA damage. For example, the DNA helicase activities of the *E. coli* UvrAB complex and of UvrD protein of *E. coli* (Oh & Grossman, 1987), as well as the DNA helicase activity of SV40 large T antigen (Gough & Wood, 1989), have been shown to be inhibited by UV radiation-induced damage to DNA. More recently, it has been demonstrated that the helicase activities of bacteriophage T4 Dda protein, *E. coli* UvrD protein (Maine et al., 1992), and *E. coli* Rep protein (Sun & Hurley, 1992) are inhibited by CC-1065 adducts. These results suggest that inhibition of enzyme activity by base damage may be a common property of DNA helicases. On the other hand, whereas Rad3 helicase activity is completely inhibited at a UV radiation dose of 1800 J/m$^2$ (Naegeli et al., 1992a), the helicase activity of the UvrAB complex is incompletely inhibited at this dose (Oh & Grossman, 1987). Furthermore, the DNA helicase activity of large T antigen is only moderately affected by UV radiation damage (Gough & Wood, 1989), and the DNA helicase activity of *E. coli* dnaB protein is unaffected by UV radiation damage at doses of up to 1800 J/m$^2$ (Oh & Grossman, 1987). Consistent with the moderate sensitivity of T antigen to UV radiation damage, it has been reported that the DNA helicase activity of this protein is not affected by base ethylation (SenGupta & Borowiec, 1992) whereas Rad3 helicase activity appears to be as sensitive to base ethylation as to UV radiation damage or bulky chemical adducts. These observations suggest that Rad3 protein may be particularly sensitive to covalent DNA damage, a view that is supported by recent studies in our laboratory which demonstrate that the DNA helicase activity of Rad3 protein is ~5 times more sensitive than the activity of UvrD protein to the presence of ~1 UV radiation-induced lesion per 1000 bases (H. Naegeli, L. Bardwell, and E. C. Friedberg, unpublished observations).

In studies in which strand selectivity for inhibition of DNA helicase activity was examined, it was reported that inhibition of Dda helicase by CC-1065 adducts is strictly DNA strand-specific (Maine et al., 1992). However, this was not the case for inhibition of the UvrD and Rep helicases by CC-1065 adducts (Maine et al., 1992; Sun & Hurley, 1992) and inhibition of the DNA helicase activity of the UvrAB complex by UV radiation damage (Oh & Grossman, 1987).

The *RAD3* gene of *S. cerevisiae* is one of six genes that are known to be absolutely required for damage-specific incision during nucleotide excision repair (Reynolds & Friedberg, 1981; Wilcox & Prakash, 1981). Several models for the possible role of a DNA helicase activity during DNA incision have been proposed, including a role in searching for damaged bases

(Oh & Grossman, 1987; Grossman & Yeung, 1990; Van Houten, 1990; Friedberg, 1991b), in loading other components of the DNA incision complex onto sites of base damage (Selby & Sancar, 1990), and in prepriming the damaged region of DNA for subsequent incision (Koo et al., 1991). The properties of Rad3 DNA helicase demonstrated in this and in earlier studies (Naegeli et al., 1992a,b) provide a basis for considering how this DNA helicase may be involved in damage-specific incision. The extraordinary sensitivity of Rad3 helicase activity to DNA strand-specific inhibition and the consequent formation of stable Rad3 protein–DNA complexes specifically with the damaged strand may serve to search for and precisely locate sites of DNA damage in the genome. When sequestered at sites of base damage, Rad3 protein may form a unique nucleoprotein structure with double-stranded DNA which is recognized by other *RAD* gene products, independent of the type of lesion, eventually generating a substrate for endonucleolytic cleavage. This model may explain how the process of damage-specific recognition during nucleotide excision repair is endowed with such extraordinary diversity.

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