The DNA Helicase and Adenosine Triphosphatase Activities of Yeast Rad3 Protein Are Inhibited by DNA Damage

A POTENTIAL MECHANISM FOR DAMAGE-SPECIFIC RECOGNITION

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Purified Rad3 protein from the yeast Saccharomyces cerevisiae is a single-stranded DNA-dependent ATPase and also acts as a DNA helicase on partially duplex DNA. In this study we show that the DNA helicase activity is inhibited when a partially duplex circular DNA substrate is exposed to ultraviolet (UV) radiation. Inhibition of DNA helicase activity is sensitive to the particular strand of the duplex region which carries the damage. Inhibition is retained if the single-stranded circle is irradiated prior to annealing to an unirradiated oligonucleotide, but not if a UV-irradiated oligonucleotide is annealed to unirradiated circular single-stranded DNA. UV irradiation of single-stranded DNA or deoxyribonucleotide homopolymers also inhibits the ability of these polynucleotides to support the hydrolysis of ATP by Rad3 protein. UV radiation damage apparently blocks translocation of Rad3 protein and results in the formation of stable Rad3 protein-UV-irradiated DNA complexes. As a consequence, Rad3 protein remains sequestered on DNA, presumably at sites of base damage. The sensitivity of Rad3 protein to the presence of DNA damage on the strand along which it translocates provides a potential mechanism for damage recognition during nucleotide excision repair and may explain the absolute requirement for Rad3 protein for damage-specific incision of DNA in yeast.

The specificity of the process of nucleotide excision repair of DNA is highly dependent on the precision of the recognition and incision of DNA containing damaged nucleotides. The biochemical basis for this specificity is poorly understood, particularly in eukaryotes. In the yeast Saccharomyces cerevisiae at least 7 distinct gene products are believed to be involved in damage-specific recognition and incision of DNA. Six of these genes, designated RAD1, RAD2, RAD3, RAD4, RAD10, and RAD14, have been identified on the basis of phenotypic characterization of mutant alleles (Friedberg, 1988). A seventh gene (designated ERCC3) has been recently identified based on sequence homology with a gene (ERCC3) required for nucleotide excision repair in human cells (Weeda et al., 1990). This genetic complexity suggests that the specific recognition of base damage and/or the incision of DNA at such sites requires multiple proteins. However, at present the functional roles of the polypeptides encoded by the 7 genes identified above are unknown.

Purification and biochemical characterization of the RAD3 gene product has demonstrated that it is a single-stranded DNA-dependent ATPase and DNA helicase (Sung et al., 1987a, 1987b; Harosh et al., 1989). Based on the paradigm of nucleotide excision repair in the prokaryote Escherichia coli (Sancar and Sancar, 1988; Van Houten, 1990), it is tempting to consider that a DNA helicase might play a role in the excision of oligonucleotide fragments generated by damage-specific incision of DNA. Alternatively, a DNA helicase might be required for preincisional events and/or incision of DNA by a specifically altering conformation at or near sites of DNA damage, as has been suggested for the UvrAB complex of E. coli (Oh and Grossman, 1989; Seeley and Grossman, 1989; Selby and Sancar, 1990; Koo et al., 1991).

Previous studies demonstrated that the DNA helicase activity of Rad3 protein was partially inhibited when a circular DNA substrate containing a 30-base pair duplex region was exposed to ultraviolet (UV) radiation (Harosh et al., 1989). In the present studies we have explored the effect of UV radiation damage on the catalytic functions of Rad3 protein in greater detail. We show here that in addition to inhibition of DNA helicase activity, UV radiation of single-stranded DNA results in inhibition of the single-stranded DNA-dependent ATPase activity. This inhibition apparently results from the sequestration of Rad3 protein on the damaged polynucleotide, presumably at sites of base damage. Additionally, when partially duplex DNA carrying photoproducts exclusively on the oligonucleotide opposite to the strand to which Rad3 protein binds and translocates was utilized as a substrate, no inhibition of DNA helicase activity was detected. These observations suggest that Rad3 protein may play an important role in searching for and locating base damage in a strand-specific manner during nucleotide excision repair in yeast.

EXPERIMENTAL PROCEDURES

Materials—M13mp18 single-stranded DNA and T4 polynucleotide kinase were purchased from New England Biolabs. DEAE-Sepharose Fast Flow, Blue Sepharose CL-6B, poly(dA), poly(dC), poly(dG), and poly(dT) were obtained from Pharmacia LKB Biotechnology Inc. Single-stranded DNA-cellulose and unlabelled nucleoside triphosphates and nucleoside diphosphates were from Sigma. M13mp19 single-stranded DNA, pUC18 plasmid DNA, λ DNA, bacterial alkaline phosphatase, and the restriction enzymes EcoRI and HindIII.
were obtained from Gibco BRL. Purified oligonucleotides were obtained from Operon Technologies Inc. [α-32P]dCTP (3000 Ci/mmole), [γ-32P]ATP (6000 Ci/mmole), and [2,8-H]ATP (32 Ci/mmole) were from Amersham Corp. Plates for thin-layer chromatography (CEL 300 polyethyleneimine/UV) were purchased from Machery-Nagel. Hydroxyapatite Bio-Gel HTP, Bio-Gel A-5m, and the protein assay kit were from Bio-Rad. Centricon-10 microcentrators were obtained from Amicon. Nitrocellulose filters (HAWP) were from Millipore Corp. Nuctrap Push Columns were from Stratagene. DNase I was purchased from Promega. Tag DNA polymerase (Amplitaq) was from Perkin-Elmer Cetus. Micrococcus luteus pyrimidine dimer-DNA glycosylase was from Applied Genetics.

Purification of Rad3 Protein—The Rad3 overexpressing yeast strain BJ-YAR was grown and Rad3 protein was purified to homogeneity as described (Harosh et al., 1989) with the following modifications. The eluate from DEAE-Sepharose chromatography (fraction II) was loaded directly onto a Blue Sepharose CL-6B column (10 × 2.5 cm) equilibrated with 0.28 M NaCl in buffer A (60 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM Na2SO4, 5 mM β-mercaptoethanol, 10% (v/v) glycerol). The column was washed with 3 bed volumes of 0.28 M NaCl in buffer A and then eluted with a linear gradient of NaCl (0.28-1.9 M) in 240 ml of buffer A. Fractions containing Rad3 protein were identified by immunoblotting (Naumovski, 1987), pooled, dialyzed against 3 changes of 50 mM NaCl in buffer A, and processed through DNA-cellulose and hydroxyapatite chromatography as described (Harosh et al., 1989). Rad3 protein was eluted from the hydroxyapatite column with 60 mM sodium phosphate and 0.1 M NaCl in buffer A. The purified enzyme migrated as a single band of 89 kDa on sodium dodecyl sulfate-polyacrylamide gels (Laemmli, 1970).

Preparation of Substrates for the DNA Helicase Assay—Partial duplex substrates for the DNA helicase assay consisted of M13mp18 single-stranded DNA annealed to either a 5' end-labeled 30-mer complementary oligonucleotide as described (Harosh et al., 1989), or to a [α-32P]-labeled 206-mer complementary oligonucleotide produced by the single-stranded polymerase chain reaction (Bednarzuk et al., 1991; see below). For annealing, 0.5-1 µg of M13mp18 DNA and an equimolar amount of oligo complementary to the labeled strand were added. The mixture was incubated at 10 mM Tris-HCl, pH 7.0, 1 mM EDTA, 100 mM NaCl, 10 mM MgCl2 (50 µl final volume) and heated at 98 °C for 10 min, followed by incubation at 72 °C (30-mer) or 82 °C (206-mer) for 20 min. Substrates were immediately purified by gel filtration through a Bio-Gel A-5m column equilibrated with 10 mM Tris-HCl, pH 7.0, 1 mM EDTA, 100 mM NaCl, at room temperature.

Polymerase chain reactions (75 µl) for the production of the [α-32P]-labeled 206-mer contained 20 mM Tris-HCl, pH 8.3, 25 mM KCl, 1.5 mM MgCl2, 0.05% Tween 20, 100 µg/ml of bovine serum albumin, 2 M each dATP, dCTP, and dTTP, 125 µCi (40 pmol) of [α-32P]dATP (3000 Ci/mmol), 100 ng of DNA 1 restriction enzyme, 2 pmol of 24-mer primer, and 5 units of Taq DNA polymerase. The amplification was performed in a DNA Thermal Cycler (Perkin-Elmer Cetus) using 55 cycles with the following set-tings: denaturation at 96 °C for 1 min, annealing at 55 °C for 1 s, and extension at 72 °C for 1 min. Initial denaturation was at 94 °C for 1 min. Following amplification the reactions were extracted with phenol/chloroform (1:1, pH 8.0) and then purified over pull columns. Typically, 30-40 ng of 206-mer were obtained at a specific radioactivity of 1.4 × 109 dpm/µg. The 206-mer oligonucleotide is complementary to nucleotides 6232-6437 of M13mp18.

UV Irradiation of DNA—Aliquots (20 µl) of DNA in 10 mM Tris, pH 7.0, 1 mM EDTA, 100 mM NaCl were placed on ice in an open Petri dish and irradiated at a dose rate of 1 J/m²·s⁻¹ using a germicidal lamp with a peak output at 254 nm.

Quantitation of Pyrimidine Dimers—The presence of pyrimidine dimers in the [α-32P]-labeled strand of the partial duplex helicase substrate was demonstrated by using the pyrimidine dimer DNA-glyco-sylase/AP endonuclease from M. luteus. Reactions (10 µl) were performed according to the manufacturer's instructions and contained 50 mM KH2PO4, pH 6.5, 40 mM NaCl, 1 mM EDTA, 1 ng of [α-32P]-labeled DNA, 100 ng of M13mp18 DNA digest, and 500 units of enzyme. After incubation at 37 °C for 60 min, reaction products were resolved by denaturing polyacrylamide gel electrophoresis (Oden and Adams, 1987) and visualized by autoradiography. Regions of the gel containing the full-length 206-mer fragment were identified and radioactivity was measured by counting Cerenkov radiations in a liquid scintillation counter. After exposure to UV radiation dose of 3600 J/m², 56% of the DNA fragments were digested. The extent of degradation in control incubations containing unirradiated DNA was less than 4%. Assuming a Poisson distribution of pyrimidine dimers in the DNA, the calculated dimer density at saturation doses was 1 dimer/249 bases of single-stranded DNA.

ATPase and DNA Helicase Assay—Hydrolysis of ATP by Rad3 protein was measured as described (Harosh et al., 1989). DNA helicase activity was determined in reaction mixtures (20 µl) containing 30 mM potassium acetate buffer, pH 5.6, 5 mM MgCl2, 1 mM dithiothreitol, 70 µg/ml of bovine serum albumin, 1 mM ATP, and the indicated amounts of helicase substrate, competitor DNA, and Rad3 protein. Reactions were stopped by the addition of 2 µl of 40 mM EDTA, 1% sodium dodecyl sulfate, and 0.1% bromphenol blue in 40% (v/v) glycerol. [α-32P]-Labeled oligonucleotide fragments were resolved by electrophoresis on polyacrylamide gels and visualized by autoradiography as described (Harosh et al., 1989; Matson, 1989). The regions of the gels containing duplex DNA and displaced fragments were removed and assayed for Cerenkov radiations in a liquid scintillation counter. Helicase activity was expressed as the percentage of displaced fragments and all values were corrected for background by subtracting the release of fragments (typically <5%) obtained in control incubations without ATP.

DNA Binding Assay—The assay used to determine binding of Rad3 protein to single-stranded DNA was adapted from Matson and Richardson (1985). Binding reactions (20 µl) contained 30 mM potassium acetate buffer, pH 5.6, 5 mM MgCl2, 1 mM dithiothreitol, 1.5 mM (in nucleotides) linear 5' end-labeled M13 single-stranded DNA and varying amounts of Rad3 protein. After incubation for 10 min at 30 °C, reaction mixtures were diluted to 1.0 ml by the addition of ice-cold 30 mM potassium acetate buffer, pH 5.6, 5.5 mM MgCl2, 1 mM dithiothreitol, and end passed through nitrocellulose filters. The filters were washed with 1.0 ml of buffer, dried, and assayed for Cerenkov radiations in a liquid scintillation counter. Protein binding was expressed as the percentage of input DNA. Background levels of DNA bound to the filters in the absence of added protein (typically <2%) were subtracted from all values. Nitrocellulose filters were pretreated by boiling in distilled water for 20 min, soaked in 0.2 M NaOH for 20 min, washed with several changes of distilled water, and stored at 4 °C in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

Preparation of Linear Single-stranded DNA M13mp18 DNA (1 µg) was incubated with 500 ng of pancreatic DNase I in 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2 for 1 min at 23 °C. The reaction was terminated by the addition of 10 mM EDTA. The linearized DNA was dephosphorylated by treatment with bacterial alkaline phosphatase, 5' end-labeled with T4 polynucleotide kinase, and purified as described (Runyon and Lohman, 1989).

RESULTS

Inhibition of Rad3 DNA Helicase Activity by UV Radiation of the Substrate—The DNA helicase activity of Rad3 protein was measured by the displacement of a [α-32P]-labeled oligonucleotide fragment annealed to complementary single-stranded M13 DNA. A previous study demonstrated partial inhibition of the Rad3 DNA helicase activity when such a substrate containing a duplex region of 30 base pairs was exposed to UV radiation (Harosh et al., 1989). In the present studies we have extended these findings using a substrate with a duplex region of 206 base pairs. Quantitative analysis of DNA helicase activity demonstrated that Rad3 protein unwound 42% of the partial duplex substrate in the control reaction. Essentially no displacement of radiolabeled oligonucleotide was detected when the substrate was exposed to 3600 J/m² of UV radiation (Fig. 1A). UV radiation-induced cross-links were not responsible for the observed inhibition since heat denaturation of the damaged substrate released >90% of the labeled oligonucleotide (data not shown). Inhibition of DNA helicase activity was UV radiation-dependent at doses between ~120 and ~900 J/m² and plateaued at ~1800 J/m² (Fig. 1B).

Strand Specificity of Inhibition of Rad3 DNA Helicase Activity—The Rad3 DNA helicase translocates on the single-
percentage of fragments released was detected by polyacrylamide gel electrophoresis as described under "Experimental Procedures" (mean values of three experiments).

After the indicated times, the reaction was stopped and the percentage of fragments released was detected by polyacrylamide gel electrophoresis as described under "Experimental Procedures" (mean values of three experiments). Open circles, unirradiated control; filled circles, UV-irradiated substrate (3600 J/m²). B, dose dependence of inhibition by UV radiation. Rad3 protein (60 ng) was incubated for 30 min with the partially duplex substrates irradiated at the indicated doses. DNA helicase activities (averages of duplicate determinations) are expressed as percentages of the oligonucleotide displacement obtained with the unirradiated control (39.9% displaced fragments).

strand to which it is bound exclusively with a 5'→3' polarity. We therefore asked whether during the unwinding of partially duplex DNA, inhibition of the helicase activity was sensitive to base damage on the unbound strand, i.e. the complementary oligonucleotide. We constructed a partial duplex substrate containing photoproducts only in the DNA fragment to be displaced (Fig. 2; substrate A). This was achieved by UV irradiating the complementary 206-mer at a dose of 3600 J/m² prior to its annealing to M13 single-stranded DNA. Using the pyrimidine dimer-specific DNA glycosylase/AP endonuclease from *M. luteus* (Grafstrom et al., 1982), we determined that 56% of the partial duplex DNA molecules contained at least one pyrimidine dimer in the annealed oligonucleotide. Control substrates were constructed by annealing either unirradiated (Fig. 2; substrate B) or UV irradiated (3600 J/m²; substrate C) M13 single-stranded DNA to the undamaged 206-mer. As shown in Fig. 2, in the presence of Rad3 protein the kinetics and extent of oligonucleotide displacement were indistinguishable with substrates A or B. As expected, the presence of UV radiation damage in substrate C inhibited Rad3 helicase activity to the same extent as that observed when the partial duplex was irradiated after annealing (Fig. 1A). These results suggest that Rad3 DNA helicase activity is uniquely sensitive to UV radiation damage in the DNA strand on which it is translocating.

**Inhibition of Rad3 ATPase Activity by UV Radiation Damage to Single-stranded DNA**—The unwinding of duplex regions of DNA by Rad3 protein is accompanied by the hydrolysis of ATP to yield ADP and P (Sung et al., 1987a, 1987b; Harosh et al., 1989). The ATPase activity is strictly dependent on the presence of single-stranded DNA or deoxyribonucleotide homopolymers (Sung et al., 1987a, 1987b; Harosh et al., 1989). UV irradiation of single-stranded M13 DNA resulted in inhibition of the Rad3 ATPase activity. After incubation for 30 min, ATP hydrolysis in the presence of M13 single-stranded DNA UV irradiated at 3600 J/m² was 51% of that measured in the presence of an equal amount of unirradiated DNA (Fig. 3A).

To explore this inhibition in greater detail we utilized a variety of synthetic homodeoxyribopolymers as substrates. Poly(dT) supported ATP hydrolysis more efficiently than did single-stranded M13 DNA (data not shown). In contrast, poly(dA), poly(dC), and poly(dG) were much less effective activators of the ATPase activity of Rad3 protein (Fig. 3B). When poly(dT) was exposed to increasing levels of UV radiation a dose-dependent inhibition of ATP hydrolysis was observed (Fig. 4). The extent of the inhibition was greater with poly(dT) than with equimolar amounts of M13 single-stranded DNA at all doses tested and was linear with respect to the UV radiation dose between ~120 and ~600 J/m² (Fig. 4). On the other hand, UV irradiation of poly(dA) had very little effect on enzyme activity (Fig. 4). These results indicate that inhibition of Rad3 ATPase activity is a function of the number of lesions in the DNA, and are consistent with several studies demonstrating that pyrimidine bases are quantitatively major targets for photochemical alterations in DNA, whereas purines are altered to a lesser extent (Friedberg, 1985).

Inhibition of the Rad3 ATPase activity by UV-irradiated polymers was also examined in a series of competition experiments. Rad3 protein was incubated with unirradiated M13 single-stranded DNA in reaction mixtures supplemented with either unirradiated or UV irradiated (3600 J/m²) poly(dT). Addition of the unirradiated homopolymer stimulated the ATPase activity of Rad3 protein and ATP hydrolysis reached...
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FIG. 3. Rad3 ATPase activity in the presence of single-stranded DNA or deoxyribonucleotide homopolymers. A, Rad3 protein (25 ng) was activated by either control (open circles) or UV-irradiated (3600 J/m²) single-stranded M13 DNA (7.6 µM, in nucleotides) (filled circles), and ATP hydrolysis (picomole) was measured as a function of time (mean values of three experiments). B, Rad3 protein (50 ng) was activated by poly(dT) (open triangles), poly(dA) (open circles), poly(dC) (open squares), or poly(dG) (filled squares), at the indicated concentrations. After 30 min reactions were stopped and ATP hydrolysis was measured as described under "Experimental Procedures" (average of duplicate determinations).

FIG. 4. Inhibition of Rad3 ATPase activity by DNA damage as a function of UV dose. Rad3 protein (25 ng) was activated by poly(dA) (circles), M13 single-stranded DNA (squares), or poly(dT) (triangles) irradiated at the indicated UV doses. Final concentrations of the polynucleotides in the reaction were 7.6 µM for poly(dT) and M13 DNA, and 76 µM for poly(dA). The incubation was stopped after 30 min and ATP hydrolysis was measured as described under "Experimental Procedures." ATPase activity (average of duplicate determinations) is expressed as a percentage of the unirradiated control.

FIG. 5. ATPase competition assay. A, Rad3 protein (20 ng) was activated by M13 single-stranded DNA (30 µM, in nucleotides) in the presence of the indicated concentrations of either unirradiated (open triangles) or UV-irradiated (3600 J/m²) poly(dT) (filled triangles). The reaction was stopped after 30 min and ATP hydrolysis was detected as described under "Experimental Procedures" (mean values of three experiments). B, control incubations without M13 single-stranded DNA. Rad3 protein (20 ng) was activated by the indicated concentrations of unirradiated (open triangles) or UV-irradiated (3600 J/m²) poly(dT) (filled triangles). The samples were incubated and analyzed as described for A (averages of duplicate determinations).

of residual ATP hydrolysis (66 pmol) was close to that observed during incubation with UV-irradiated poly(dT) alone (40 pmol) (Fig. 5, A and B).

These results indicate that the inhibition of ATPase activity is not due to a reduced binding affinity of Rad3 protein for the UV-irradiated polymer relative to the unirradiated polymer or single-stranded DNA. On the contrary, they suggest that the protein might bind preferentially to UV-irradiated poly(dT), resulting in enzyme inhibition due to sequestration of Rad3 protein at or near sites of DNA damage.

Binding of Rad3 Protein to Single-stranded DNA—In order to directly examine the binding of Rad3 protein to UV-irradiated single-stranded DNA, we incubated Rad3 protein with linear 5’ end-labeled M13 DNA and analyzed the formation of protein-DNA complexes by retention of DNA on nitrocellulose filters. Addition of Rad3 protein to unirradiated single-stranded DNA led to maximal retention of ~80% of the input DNA on nitrocellulose filters (Fig. 6). Half-maximal retention of input DNA was obtained at a molar ratio of Rad3 protein:M13 DNA of 58:1. This requirement for excess protein may reflect a low percentage of active enzyme molecules or, more likely, a low efficiency of binding of DNA-protein complexes to nitrocellulose filters (Matson and Richardson, 1985). Equilibrium binding was achieved after 10 min of incubation, since longer incubations did not significantly increase the fraction of bound DNA. ATP was not required for the formation of Rad3 protein-DNA complexes (data not shown). However, the triphosphate was routinely included in all reactions.

a maximum level of 352 pmol during a 30-min incubation (Fig. 5A).

The same level of ATPase activity was obtained in a control reaction in the presence of unirradiated poly(dT) alone (Fig. 5B). Addition of increasing amounts of UV-irradiated poly(dT) to reactions with unirradiated single-stranded DNA resulted in reduced hydrolysis of ATP (Fig. 5A). At a poly(dT):single-stranded DNA molar ration of 1:1, the level
control of filters and conditions necessary to avoid binding of naked single-stranded DNA to filters are described under “Experimental Procedures.” DNA retained on filters (mean values of three experiments) is expressed as the percentage of total input DNA.

FIG. 6. Rad3 protein binding to single-stranded DNA. The indicated amounts of Rad3 protein were mixed with unirradiated control (open squares) or UV-irradiated (3600 J/m²) linear 5’ end-labeled M13 single-stranded DNA (1.5 nM in nucleotides) (filled squares), and incubated in the presence of ATP. Reactions were terminated after 10 min and samples were loaded onto nitrocellulose filters as described under “Experimental Procedures.” Pretreatment of filters and conditions necessary to avoid binding of naked single-stranded DNA to filters are described under “Experimental Procedures.” DNA retained on filters (mean values of three experiments) is expressed as the percentage of total input DNA.

FIG. 7. Rates of dissociation of Rad3 protein-DNA complexes. Rad3 protein (5 ng) was incubated with linear 5’ end-labeled M13 single-stranded DNA (1.5 nM in nucleotides) and ATP. The DNA substrate was either unirradiated (open squares) or UV-irradiated at 3600 J/m² (filled squares). After 10 min a 20-fold excess of unlabeled and unirradiated M13 single-stranded DNA was added and incubations were continued for the indicated time periods. The reaction mixtures were loaded onto nitrocellulose filters as described under “Experimental Procedures.” DNA retained on the filters is expressed as the percentage of total input DNA (mean values of four experiments).

Binding of Rad3 protein to DNA was unaffected by the presence of UV radiation-induced damage (Fig. 6). Even at the highest UV dose used (3600 J/m²) we observed the same amount of DNA retention on filters. Concomitant measurement of ATP hydrolysis confirmed that UV irradiation of the linear substrate used in these experiments produced the same inhibitory effect on Rad3 ATPase activity as the circular DNA used in the experiments described above. These results provide direct confirmation that inhibition of the ATPase activity of Rad3 protein is not caused by reduced binding of protein to the UV-irradiated DNA substrate.

Dissociation Kinetics of Rad3 Protein-DNA Complexes—When challenged with an excess of cold competing single-stranded M13 DNA, Rad3 protein rapidly dissociated from radiolabeled M13 DNA (Fig. 7). After 30 min of incubation, 8.0% of the input DNA (corresponding to 11.3% of the initial amount of Rad3-DNA complexes) was retained on nitrocellulose filters. The majority of the complexes dissociated during the first 10 min after the addition of competing DNA (Fig. 7), yielding a calculated half-life of the complex of 3.9 min. When competitor DNA was added to incubations of Rad3 protein with UV-irradiated M13 DNA, Rad3 protein initially dissociated from the radiolabeled irradiated DNA at a similar rate (Fig. 7). However, 2 min after the addition of the competitor, DNA turnover of the protein was significantly reduced and a considerable fraction of the Rad3-UV-irradiated DNA complexes remained stable (Fig. 7). During the slower dissociation phase the half-life for dissociation of Rad3-UV-irradiated DNA complexes was calculated at 84.0 min. These results indicate that Rad3 protein remains tightly bound to the damaged substrate and, together with the observed inhibition of DNA helicase activity (Fig. 1), suggest that sites of UV radiation damage block the translocation of Rad3 protein along single-stranded DNA.

Sequestration of Rad3 Protein on UV-damaged Single-stranded DNA or Poly(dT)—To determine the fraction of Rad3 protein sequestered at sites of UV radiation damage in single-stranded DNA, we incubated purified Rad3 protein with varying amounts of unirradiated or UV-irradiated M13 single-stranded DNA for 10 min and then added a substrate for DNA helicase activity, consisting of circular single-stranded M13 DNA annealed to a complementary 5’ end-labeled 30-mer primer. After incubation for a further 30 min, the reaction was stopped and helicase activity was measured. We concurrently determined that under standard assay conditions in the absence of a competing substrate, DNA helicase activity was linear with respect to Rad3 protein concentration between 5 and 100 ng of protein. This helicase competition assay therefore provided a convenient method for quantitating the fraction of active Rad3 protein that remained sequestered on the UV-irradiated single-stranded DNA.

A representative result is shown in Fig. 8A. As expected,
helicase activity was progressively reduced as a function of added competitor single-stranded DNA. At all concentrations tested, UV-irradiated (3600 J/m²) M13 DNA was a stronger competitor than the unirradiated DNA. For example, when 4 μM M13 DNA was used as the competitor (Fig. 8A, lane 7) helicase activity was 70.2% of that measured in its absence (Fig. 8A, lane 2). On the other hand, 4 μM UV-irradiated DNA reduced helicase activity to 30.6% of the control reaction (Fig. 8A, lane 8). Hence, an additional 39.6% of the active Rad3 enzyme molecules apparently remained sequestered on the UV-irradiated competitor DNA, and were not available for unwinding of the DNA helicase substrate. Assuming that all enzyme molecules were active, we calculated that 249 ± 9.3 fmol of Rad3 protein were sequestered on UV-irradiated M13 DNA in reactions containing 4 μM UV-irradiated M13 competitor DNA. This translates to an average of 1 molecule of Rad3 protein sequestered per 321.3 (±23.2) nucleotides of UV-irradiated M13 DNA.

This phenomenon was even more striking when poly(dT) was used for competition. Calculations from the data shown in Fig. 8B indicate that on the average 1 enzyme molecule of Rad3 protein was sequestered for every 151.9 (±17.9) nucleotides. This effect was not observed in the presence of UV-irradiated poly(dA) (Fig. 8B), presumably reflecting the lower number of photochemical alterations that can be induced by UV irradiation of purines. The extent of the sequestration of Rad3 protein was essentially linear with respect to the UV radiation dose in the range 120–900 J/m² and plateaued around 1800 J/m² (Fig. 9).

**DISCUSSION**

Rad3 protein of *S. cerevisiae* is a DNA-dependent ATPase/ DNA helicase (Sung et al., 1987a, 1987b; Harosh et al., 1989). The ATPase activity utilizes exclusively single-stranded DNA; essentially no hydrolysis of ATP is detected in the presence of duplex DNA (Sung et al., 1987a, 1987b; Harosh et al., 1989). The requirement for single-stranded DNA for nucleoside and deoxyribonucleoside triphosphate hydrolysis is central to the mechanism of unwinding of duplex DNA by all known DNA helicases (Matson and Kaiser-Rogers, 1990). It is generally assumed that the energy released during the hydrolysis of triphosphates is utilized in the helicase reaction, although little is known about the detailed mechanism of DNA unwinding (Matson and Kaiser-Rogers, 1990).

Studies on bacteriophage T7 G4 protein, which like Rad3 protein is a DNA helicase with a strict 5′→3′ polarity with respect to the single strand to which it is bound, suggest that the hydrolysis of nucleoside triphosphates facilitates the processive unidirectional translocation of the protein along single-stranded DNA (Matson and Richardson, 1983; 1985). Similar conclusions were reached by Brown and Romano (1989), who hypothesized that hydrolysis of nucleoside triphosphate by phage T7 G4 protein results in a conformational change in the protein when bound to single-stranded DNA and that this change is required for its translocation.

We previously reported that the displacement of a 30-mer complementary oligonucleotide from M13 single-stranded circular DNA by Rad3 protein was significantly reduced if the substrate was previously exposed to UV irradiation (Harosh et al., 1989). Simpler results were reported for the DNA helicase activity of *E. coli* UvrA/UvrB protein complexes (Oh and Grossman, 1987, 1989). In the present experiments we observed more complete inhibition of Rad3 DNA helicase activity with a UV-irradiated substrate containing a longer partially duplex region. The longer length of the oligonucleotide in this substrate is expected to increase the probability of generating photoproducts in the duplex region and this might explain the quantitative differences between the two studies.

A novel feature of the present experiments is the observation that inhibition of the Rad3 DNA helicase activity by UV radiation damage is strand specific and is effected only in the presence of damage on the strand on which the protein is translocating. These results are in contrast to those previously reported with the DNA helicase activity of the *E. coli* UvrAB protein, in which it was observed that irradiation of either the entire partially duplex DNA substrate or just the complementary oligonucleotide prior to annealing resulted in partial inhibition of helicase activity (Oh and Grossman, 1987). However, it should be noted that the biochemical properties of the yeast Rad3 and the *E. coli* UvrAB helicases are not identical. In particular, the *E. coli* UvrAB complex only unwinds short DNA duplex regions and acts in a distributive mode (Oh and Grossman, 1989).

The observation that UV radiation of single-stranded DNA or poly(dT) inhibits the hydrolysis of ATP by Rad3 protein is consistent with the inhibition of DNA helicase activity. A similar phenomenon was recently reported by Brown and Romano (1989), who observed inhibition of the dTTPase activity of phage T7 G4 protein in the presence of single-stranded DNA containing benzo[alpyrene adducts. These investigators concluded that 'G4 protein is sequestered at benzo[alpyrene adducts. Our results support such a phenomenon. We demonstrated that in the presence of unirradiated single-stranded DNA, Rad3 protein forms short-lived DNA-protein complexes with a half-life of 3.9 min. However, in the presence of UV-irradiated DNA a significant fraction of Rad3 DNA complexes are considerably more stable, with a half-life of 84 min. Additionally, UV-irradiated single-stranded DNA competes more efficiently for DNA helicase activity than does unirradiated single-stranded DNA, suggesting that Rad3 protein remains sequestered on UV-irradiated DNA, presumably at sites of base damage.

The mechanism by which polynucleotide damage inhibits the nucleoside triphosphatase activity of DNA helicases such as the Rad3 and phage T7 G4 proteins is not clear. We have observed that single-stranded RNA is totally ineffective as a polynucleotide activator for ATP hydrolysis by Rad3 protein. This observation, together with the observation that different deoxyribonucleotide homopolymers support ATP hydrolysis

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1. H. Naegeli, L. Bardwell, and E. C. Friedberg, manuscript in preparation.
by Rad3 protein with different efficiencies, suggests that the conformational change(s) in the protein that are required for ATP hydrolysis are exquisitely sensitive to the chemistry of the polynucleotide. Hence, damage to single-stranded DNA might inhibit the hydrolysis of nucleoside triphosphates, and RNA may be “read” by Rad3 protein as a form of damaged DNA. Additionally, if, as has been suggested by Brown and Romano (1989) for phage T7 G4 protein, the power stroke for translocation along single-stranded DNA is actually the exchange of nucleoside triphosphate for the diphosphate, continued translocation may become rate-limiting for adopting a conformation necessary for hydrolysis of the triphosphate.

Regardless of the precise mechanism(s) whereby DNA damage inhibits nucleotide hydrolysis and translocation of DNA helicases along single-stranded regions of DNA, this phenomenon potentially offers a highly specific mechanism for locating sites of base damage in DNA. Hence, Rad3 protein may play a fundamental role in damage-specific recognition during nucleotide excision repair. Following arrested translocation of Rad3 protein at sites of damage, other proteins required for DNA incision may bind to Rad3-DNA complexes, eventually generating a conformational state required for endonucleolytic cleavage of DNA. Two attractive features of this model immediately come to mind. One is that the model readily accommodates the strand specificity associated with the excision of damaged nucleotides. Second, the model accommodates the observation that nucleotide excision repair operates on a wide variety of chemically distinct types of base damage. Indeed, recent studies in E. coli suggest that lesions as diverse as pyrimidine dimers and abasic sites are recognized by the UvrABC endonuclease (Lin and Sancar, 1989; Van Houten, 1990). The strand-specific effect of other types of base damage in DNA on the Rad3 DNA helicase and ATPase activities is currently under investigation.

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