Purification of Rad1 Protein from *Saccharomyces cerevisiae* and Further Characterization of the Rad1/Rad10 Endonuclease Complex†

Alan E. Tomkinson,*,‡ A. Jane Bardwell,§ Nancy Tappe,§ William Ramos,‡ and Errol C. Friedberg§

Laboratory of Molecular Pathology, The University of Texas Southwestern Medical Center, Dallas, Texas 75235, and Institute of Biotechnology, Center for Molecular Medicine, The University of Texas Health Science Center at San Antonio, 15355 Lambda Drive, San Antonio, Texas 78245

Received December 7, 1993; Revised Manuscript Received February 22, 1994®

ABSTRACT: The yeast recombination and repair proteins Rad1 and Rad10 associate with a 1:1 stoichiometry to form a stable complex with a relative molecular mass of 190 kDa. This complex, which has previously been shown to degrade single-stranded DNA endonucleolytically, also cleaves supercoiled duplex DNA molecules. In this reaction, supercoiled (form I) molecules are rapidly converted to nicked, relaxed (form II) molecules, presumably as a result of nicking at transient single-stranded regions in the supercoiled DNA. At high enzyme concentrations, there is a slow conversion of the form II molecules to linear (form III) molecules. The Rad1/Rad10 endonuclease does not preferentially cleave UV-irradiated DNA and has no detectable exonuclease activity. The nuclease activity of the Rad1/Rad10 complex is consistent with the predicted roles of the *RAD1* and *RAD10* genes of *Saccharomyces cerevisiae* in both the incision events of nucleotide excision repair and the removal of nonhomologous 3′ single strands during intrachromosomal recombination between repeated sequences. In these pathways, the specificity and reactivity of the Rad1/Rad10 endonuclease will probably be modulated by further protein–protein interactions.

The *RAD1* and *RAD10* genes of the yeast *Saccharomyces cerevisiae* are required both for nucleotide excision repair (NER)[1] of DNA and for specialized forms of mitotic recombination (Aguilera & Klein, 1989; Higgins et al., 1983; Reynolds et al., 1985, 1987; Schiestl & Prakash, 1989, 1990; Thomas & Rothstein, 1989). Mutational inactivation of either gene results in a phenotype of severe sensitivity to UV[1] radiation and defective incision of DNA in UV-irradiated cells (Prakash, 1977; Reynolds & Friedberg, 1981; Unrau et al., 1971). These observations have led to the suggestion that the Rad1 and Rad10 proteins participate in the specific incision of damaged DNA during NER.

The *RAD1* and *RAD10* genes encode polypeptides with predicted molecular weights of 126 200 and 24 300, respectively (Reynolds et al., 1985, 1987). Recent studies using both *in vitro* (Bailly et al., 1992; Bardwell et al., 1992) and *in vivo* (Bardwell et al., 1993) systems have demonstrated that these polypeptides form a stable and specific complex in the absence of DNA. Additionally, we recently reported that the incubation of single-stranded circular DNA with a mixture of purified Rad1 and Rad10 proteins, but not either protein alone, results in endonucleolytic degradation of the DNA (Tomkinson et al., 1993). This observation provides support for the postulated role of these proteins in DNA incision during NER. The ability to degrade single-stranded DNA is also consistent with the postulated role of the *RAD1* (Fishman-Lobell & Haber, 1992) (and presumably the *RAD10*) gene in recombinational events between repeated sequences.

The purification of Rad10 protein to apparent physical homogeneity has been previously documented (Bardwell et al., 1990; Sung et al., 1992). In the present studies, we report the purification of Rad1 protein to >90% homogeneity following overexpression of this protein in yeast cells, and present a strategy that significantly improves the yield of purified Rad10 protein. We also show that Rad1 and Rad10 proteins form a complex with 1:1 stoichiometry, and that in addition to single-stranded DNA, this complex nicks supercoiled DNA, presumably at single-stranded regions in such molecules.

EXPERIMENTAL PROCEDURES

Overexpression of Rad1 Protein in E. coli. A *Bam*HI fragment containing the *S. cerevisiae RAD1* gene (Yang, 1987) was subcloned in the *Bam*HI site of the *Escherichia coli* expression vector pDR450 (Pharmacia) to generate the plasmid ptac*RAD1*. After induction of *RAD1* expression with IPTG,[1] cells were harvested and lysed prior to extraction of Rad1 from the insoluble fraction with 1% sarkosyl/25 mM sodium phosphate (pH 7.5). After separation by SDS[1]–polyacrylamide gel electrophoresis (Laemmli, 1970) and Coomassie blue staining, Rad1 protein was identified as a polypeptide with a relative molecular mass of 150 kDa in lysates of cells overexpressing ptac*RAD1*, but not in comparable lysates from cells carrying pDR540.

Production and Affinity Purification of Rad1 Antisera. Rabbit polyclonal antiserum was raised against Rad1 protein overexpressed in *E. coli* and affinity-purified by essentially the same procedure previously reported for the production of Rad10-specific antibodies (Bardwell et al., 1990).

Growth of Yeast Strains for Protein Purification. Rad10 protein was purified from the *S. cerevisiae* strain SF657-2D (*MATa pep4-3 leu2-3,112 his4 ura3-52 gal2*), transformed with plasmid pG12-*RAD10* (Bardwell et al., 1990). This strain was grown in a 60-L New Brunswick fermentor at 30 °C, pH

† These studies were supported by U.S. Public Health Service Grants CA12428 (E.C.F.) and RR07187-14 (A.E.T.) from the Department of Health and Human Services.

* Correspondence should be addressed to this author at the Institute of Biotechnology, Center for Molecular Medicine, The University of Texas Health Science Center at San Antonio, 15355 Lambda Dr., San Antonio, TX 78245.

‡ The University of Texas Health Science Center at San Antonio.

§ The University of Texas Southwestern Medical Center.

® Abstract published in *Advance ACS Abstracts,* April 1, 1994.

[1] Abbreviations: DTT, dithiothreitol; FPLC, fast protein liquid chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; NER, nucleotide excision repair; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; SDS, soduim dodecyl sulfate; UV, ultraviolet.

0006-2960/94/0433-5305$04.50/0

5.5, in yeast minimal medium (1.7 g/L yeast nitrogen base, 20 g/L sucrose, and 5 g/L ammonium sulfate) supplemented with histidine (20 mg/L) and uracil (20 mg/L). Cells were harvested by centrifugation at OD_{600} = 1.2. Approximately 200 g (wet weight) of cells was obtained from a 60-L culture.

Rad1 protein was purified from the *S. cerevisiae* strain BJ2168Δ*RAD10* (Weiss & Friedberg, 1985), transformed with plasmid pG12-*RAD1*. This strain was grown in yeast minimal medium supplemented with tryptophan (20 mg/L) and uracil (20 mg/L). After growth in a 60-L New Brunswick fermentor at 30 °C, pH 5.5, cells were harvested at OD_{600} = 0.9. Approximately 200 g (wet weight) of cells was obtained from a 60-L culture. In contrast to the overexpression of Rad10 protein, overexpression of Rad1 protein from the plasmid pG12-*RAD1* was variable in both *RAD10* deletion and wild-type genetic backgrounds. Cells freshly transformed with plasmid DNA, i.e., not stored or frozen, gave the best levels of Rad1 overexpression.

Purification of Rad10 Protein. A lysate was prepared from a frozen cell pellet (120 g) as described previously (Tomkinson *et al.*, 1992). Protein concentrations were estimated by the method of Bradford (1976). Rad10 protein in the cleared lysate (7.2 g of protein in 450 mL) was precipitated with ammonium sulfate (Sung et al., 1992) and resuspended in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, 10 mM β-mercaptoethanol, 10% glycerol, 1 mM PMSF,[1] 1 mM benzamidine hydrochloride, 0.5 μg/mL chymostatin and leupeptin, 0.7 μg/mL pepstatin, and 0.4 μg/mL aprotinin (buffer A). The suspension was dialyzed against 2 L of buffer A for 2 h and then against 4 L of the buffer overnight.

After centrifugation to remove insoluble debris, the dialysate (3 g of protein in 300 mL) was applied to a 5 × 20 cm DEAE-cellulose (Whatman) column preequilibrated with buffer A. Pass-through fractions (1.6 g of protein in 450 mL) were applied to a 2.6 × 35 cm phophocellulose (Whatman) column preequilibrated with buffer A. Bound proteins were eluted with a 1-L gradient from 50 mM to 0.5 M NaCl in buffer A. Rad10-containing fractions were detected by immunoblotting, pooled (50 mg of protein in 130 mL), and further purified by Blue Sepharose (Pharmacia) column chromatography (Bardwell et al., 1990). Fractions containing Rad10 protein were pooled (12 mg of protein in 60 mL) and dialyzed against 25 mM Hepes[1]–NaOH (pH 7.6), 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT,[1] and 10% glycerol (buffer B). The dialysate was divided in half and further purified separately (the protein yields represent the sum of the two identical fractionations).

Dialysates were applied to an FPLC[1] Mono S HR 5/5 column (Pharmacia). Bound proteins were eluted with a 20-mL gradient from 50 mM to 0.5 M NaCl in buffer B. Rad10-containing fractions that eluted at ~240 mM NaCl were pooled (4 mg of protein in 5 mL) and applied to a HiLoad 1.6 × 60 cm Superdex 75 column preequilibrated with buffer B containing 0.5 M NaCl. Fractions containing Rad10 protein were pooled (2.5 mg of protein in 16 mL), dialyzed against buffer C (buffer B at pH 7.8), and applied to an FPLC Mono S HR 5/5 column. Proteins were eluted with a 20-mL gradient from 50 mM to 0.5 M NaCl in buffer C. The peak fractions of Rad10 protein (2 mg of protein in 2 mL) were >90% homogeneous as judged by denaturing PAGE.[1] Rad10 protein has been stored on ice for 4 weeks and for 6 months at −80 °C without significant loss of activity.

Purification of Rad1 Protein. A cleared lysate (2.8 g of protein in 410 mL) was prepared from the cell pellet (110 g), and proteins were fractionated with ammonium sulfate as described above. The ammonium sulfate pellet was resuspended in buffer A, and insoluble material was removed by centrifugation after dialysis against buffer A. The cleared dialysate (1.3 g in 200 mL) was applied to a DEAE-cellulose column (2.6 × 30 cm). After the column was washed with buffer A, bound protein was eluted with a 1-L salt gradient (50 mM–1 M NaCl) in buffer A. Fractions containing Rad1 protein eluted at ~275 mM NaCl and were detected by immunoblotting (Tomkinson et al., 1993). These fractions (125 mg of protein in 140 mL) were pooled, dialyzed against buffer A, and applied to a heparin–Sepharose (Pharmacia) column (2.6 × 8 cm). Bound proteins were eluted with a 400-mL gradient (50–750 mM NaCl) in buffer A. Rad1 fractions which eluted at about 0.55 M NaCl were pooled (14 mg of protein in 65 mL) and dialyzed against 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, 0.5 mM DTT, and 10% glycerol (buffer D). The dialysate was divided in half and further purified separately. (The protein yields represent the sum of the two identical fractionations). Proteins were applied to an FPLC Mono Q HR 5/5 column (Pharmacia). Bound proteins were eluted with a 20-mL gradient (50–750 mM NaCl) in buffer D. Fractions containing Rad1 protein eluted at about 0.4 M NaCl and were pooled (5 mg of protein in 4 mL) and applied to a HiLoad 1.6 × 60 cm Superdex 200 column preequilibrated with buffer D containing 0.75 M NaCl. Rad1-containing fractions (3 mg, 14 mL) were dialyzed against buffer D and concentrated on the FPLC Mono Q column as described above. The peak fractions of Rad1 (1.2 mg of protein in 2.4 mL) were >90% homogeneous as judged by denaturing gel electrophoresis. Rad1 protein has been stored at −80 °C for 6 months without significant loss of activity.

Immunoblotting. Proteins were separated by SDS–PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes. Antibody–antigen complexes were detected by enhanced chemiluminescence (Amersham).

Polynucleotide Substrates. PM2 phage DNA was prepared as described by Espejo and Canelo (1968) as modified by Kuhnlein et al. (1976). Supercoiled DNA was converted to the relaxed closed circular form with wheat germ topoisomerase I (Promega) or to the linear duplex form with *Msp*I restriction enzyme (Promega). Phage fd [^{3}H]thymidine-labeled DNA (26 000 cpm/nmol of nucleotide) was prepared as previously described (Goldmark & Linn, 1970). *E. coli* [^{3}H]thymidine-labeled DNA (5100 cpm/nmol of nucleotide) was prepared as described by Lehman (1960). φX174 RF DNA was purchased from Gibco/BRL. All polynucleotide concentrations are expressed as nucleotide residues.

Alkaline Sucrose Gradients. Reaction mixtures (50 μL) were mixed with 5 μL of 0.5 M EDTA and then with 50 μL of 500 mM NaOH, 1.8 M NaCl, and 10 mM EDTA. The samples were then layered onto a 5-mL 5–25% linear sucrose gradient containing 250 mM NaOH, 900 mM NaCl, and 5 mM EDTA. Gradients were centrifuged at 40 000 rpm for 4 h in a Beckman SW50.1 rotor at 4 °C. After sedimentation, fractions were collected from the bottom of the tube. Sedimentation of poly(dA) (average length 540 nucleotides, Pharmacia) was detected by the absorbancy at 260 nm. Fractions from gradients containing radioactivity were neutralized by the addition of 100 mM Tris-HCl (pH 8.0)/0.17 M HCl prior to liquid scintillation counting.

RESULTS

Purification of Rad10 Protein. We have modified previously published protocols (Bardwell et al., 1990; Sung et al., 1992) for the purification of Rad10 protein (see Experimental Procedures), thereby generating a procedure that reproducibly results in an ~20% yield of Rad10 protein that is >90% homogeneous (Figure 1). Rad10 protein purified by this

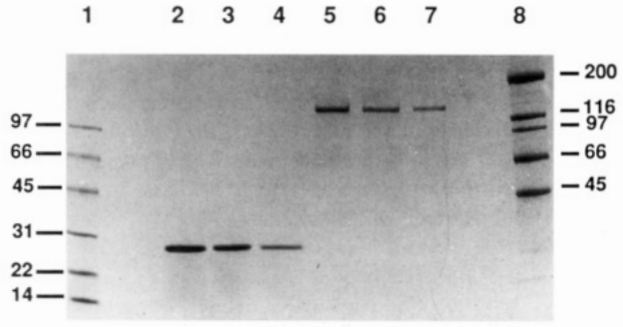

FIGURE 1: Size of purified Rad10 and Rad1 proteins as measured by SDS–polyacrylamide gel electrophoresis. Rad10 and Rad1 proteins were purified as described under Experimental Procedures. The purified fractions were electrophoresed through a denaturing 4–20% polyacrylamide gradient gel (BioRad), and proteins were detected by staining with Coomassie blue. Lane 1, low-range molecular mass standards (BioRad); lane 2, 630 ng of Rad10 protein; lane 3, 420 ng of Rad10 protein; lane 4, 210 ng of Rad10 protein; lane 5, 585 ng of Rad1 protein; lane 6, 390 ng of Rad1 protein; lane 7, 195 ng of Rad1 protein; lane 8, high-range molecular mass standards (BioRad).

scheme catalyzes the annealing of homologous DNA single strands (data not shown) as described previously (Sung et al., 1992). The scheme used by Sung et al. (1992) resulted in the purification of Rad10 protein with a yield similar to that observed in the present study, but in significantly lower amounts per gram of starting material (1.3 μg/g compared to 17 μg/g). This difference suggests that the level of Rad10 is at least 10-fold greater in strain SF657-2D transformed with plasmid pG12-*RAD10* than in strain CMY135 transformed with plasmid pSUC8 (Sung et al., 1992).

Purification of Rad1 Protein. The observation that the Rad1 and Rad10 proteins form a specific and stable complex (Bardwell et al., 1992) suggested the possibility of purifying Rad1 protein by affinity chromatography using immobilized Rad10 protein. In initial experiments, we examined the specific binding of radiolabeled *in vitro*-translated Rad1 protein (Bardwell et al., 1992) to Rad10 protein that was covalently attached to an inert matrix. Full-length (but not truncated) Rad1 protein did indeed bind to Rad10–Affigel beads and not to similarly prepared BSA beads (data not shown). The failure of the Rad10 beads to bind truncated forms of Rad1 is consistent with the demonstration that the C-terminal portion of Rad1 is required for the interaction with Rad10 (Bardwell et al., 1992, 1993). Bound Rad1 protein could only be recovered following elution of the beads in the presence of 1% SDS at 70 °C. The harsh conditions required to dissociate the Rad1/Rad10 complex precluded the use of this affinity strategy to purify biochemically active Rad1 protein.

In subsequent experiments, Rad1 protein was overproduced in a yeast strain that was deleted of the *Rad10* gene. The rationale for this approach was to eliminate endogenous Rad10 protein, which could potentially complex with Rad1 protein and thereby substantially reduce or even eliminate its yield. The purification of Rad1 (see Experimental Procedures) was monitored by an immunoblotting assay using affinity-purified polyclonal antiserum raised against Rad1 protein overexpressed in *E. coli*. Rad1 protein purified by this scheme was >90% homogeneous as judged by denaturing gel electrophoresis (Figure 1).

Purified Rad1 Protein Complexes with Rad10 Protein. The purification of Rad1 protein using the immunoblotting assay described above provides no indication about the functional integrity of the protein. In order to evaluate this parameter, we examined several known biological attributes

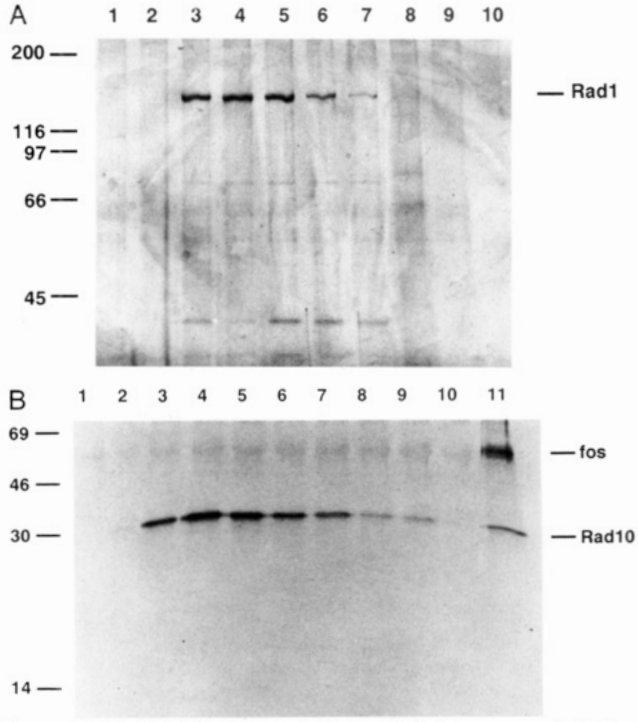

FIGURE 2: Purified Rad1 protein directs the coimmunoprecipitation of *in vitro*-translated Rad10 protein. A side fraction containing Rad1 protein (~20 μg) from the gel filtration step (Experimental Procedures) was applied to an FPLC 5/5 Mono Q column, which was eluted with a 30-mL NaCl gradient (50–750 mM). Elution of Rad1 protein was detected by monitoring the absorbancy at 280 nm. (A) Aliquots (10 μL) of consecutive fractions containing Rad1 protein were electrophoresed through a 7.5% denaturing polyacrylamide gel, and protein was detected by silver staining (lanes 1–10). Molecular mass standards are shown on the left. (B) Rad10 and Fos proteins were independently translated *in vitro* in the presence of [^{35}S]-methionine as described (Bardwell et al., 1992). Labeled polypeptides were detected by autoradiography after separation through a 15% denaturing polyacrylamide gel (lane 11). The labeled proteins (26 ng of Rad10 and 33 ng of Fos) were incubated with the Mono Q fractions containing Rad1 protein (2 μL) shown in (A). Following immunoprecipitation with anti-Rad1 antibodies as described (Bardwell et al., 1992), the labeled polypeptides were resolved by denaturing gel electrophoresis and detected by autoradiography (lanes 1–10). Molecular mass standards are shown on the left.

of Rad1 protein. In Figure 2, we show that the 150-kDa polypeptide, previously identified as Rad1 protein by immunoblotting (Tomkinson et al., 1993), interacts specifically with labeled Rad10 protein. In this experiment, partially purified Rad1 protein was eluted from an FPLC Mono Q column and was detected by monitoring the absorbancy at 280 nm and by silver staining after denaturing gel electrophoresis (Figure 2A). Fractions from the gradient were incubated with radiolabeled *in vitro*-translated Rad10 and Fos proteins (Bardwell et al., 1992). Following incubation, anti-Rad1 antibody was added, and immunoprecipitates were harvested, subjected to denaturing gel electrophoresis, and analyzed by autoradiography. Immunoprecipitation of each of the fractions containing Rad1 protein resulted in the exclusive coimmunoprecipitation of Rad10 protein and not Fos protein (Figure 2B). Hence, Rad1 protein purified by the procedure described here is able to bind specifically to Rad10 protein.

In order to determine the stoichiometry of this binding, we examined the gel filtration behavior of Rad1 protein, Rad10 protein, and mixtures of the two preincubated under conditions known to result in complex formation. When purified Rad1 protein was subjected to gel filtration, a single protein peak (detected by the absorbancy at 280 nm) eluted with a relative molecular mass of 160 kDa (Figure 3A). After denaturing gel electrophoresis, we detected a 150-kDa polypeptide by

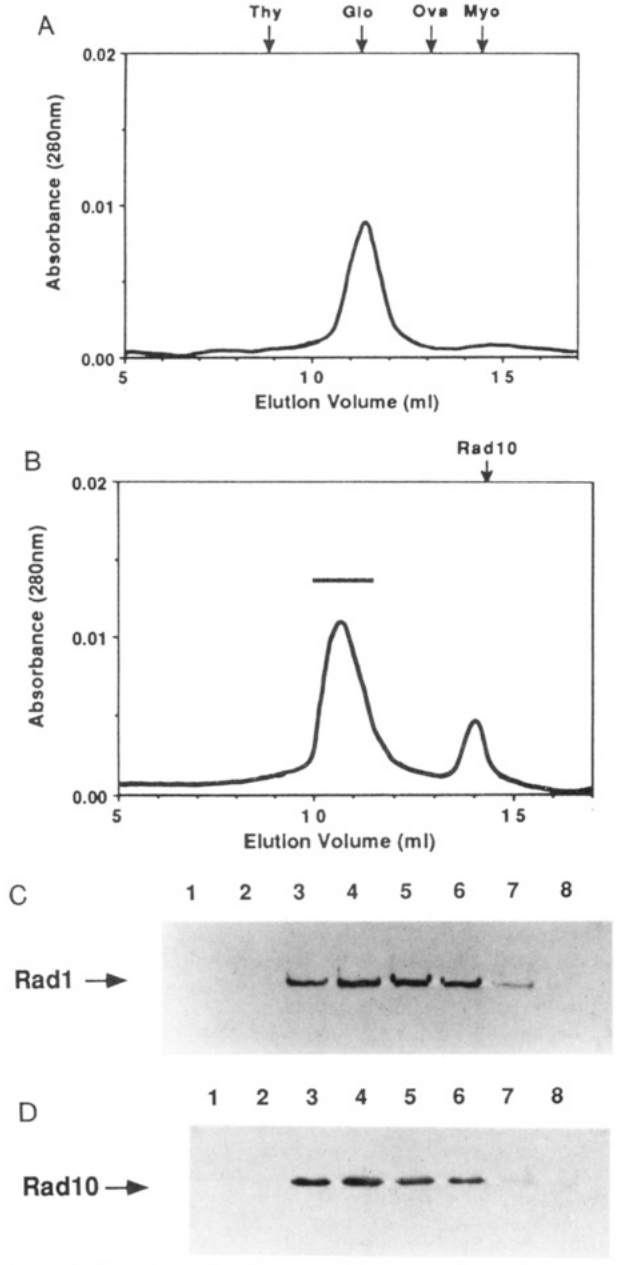

FIGURE 3: Relative molecular mass of Rad1 protein, Rad10 protein, and the Rad1/Rad10 complex as measured by gel filtration. (A) Purified Rad1 protein (30 μg, 240 pmol) was loaded in a final volume of 100 μL onto a FPLC Superose 12 gel filtration column that had been preequilibrated with 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and 10% glycerol. The sample was then eluted with the above buffer at a flow rate of 0.3 mL/min. The protein elution profile detected by monitoring the absorbancy at 280 nm is shown. Molecular mass standards (thyroglobulin, 670 kDa; γ-globulin, 158 kDa; ovalbumin, 44 kDa; and myoglobin, 17.5 kDa; from BioRad) were separated under identical conditions, and their elution positions are indicated. (B) Rad1 protein (28 μg, 221 pmol) and Rad10 protein (8.7 μg, 355 pmol) were mixed and preincubated at 30 °C for 15 min in a final volume of 100 μL. After being loaded onto the gel filtration column, proteins were eluted as described above. The protein elution profile shown was detected by monitoring the absorbancy at 280 nm. Fractions (300 μL, 1–8) from the column eluate corresponding to the part of the elution profile indicated by the line above the peak were further analyzed. (C) Proteins were separated through a 7.5% denaturing polyacrylamide gel and transferred to nitrocellulose. After incubation of the membrane with Rad1 antibody, Rad1 protein was quantitatively detected by enhanced chemiluminescence. (D) Proteins were separated through a 15% denaturing polyacrylamide gel and transferred to nitrocellulose. After incubation of the membrane with Rad10 antibody, Rad10 protein was quantitatively detected by enhanced chemiluminescence.

staining and by immunoblotting with Rad1 antibody that coeluted with the single peak shown in Figure 3A (data not shown). The relative molecular mass of 160 kDa is significantly greater than the molecular weight of 126 200 predicted from the *RAD1* open reading frame (Reynolds et al., 1987), suggesting that Rad1 protein has an asymmetric shape which leads to anomalous behavior during gel filtration. In similar gel filtration experiments, homogeneous Rad10 protein eluted with a relative molecular mass of 25 kDa (data not shown), consistent with the calculated molecular weight of this protein (Reynolds et al., 1985).

When purified Rad1 and Rad10 proteins were preincubated at a molar ratio of 1:1.6 and then subjected to gel filtration, the molecular mass of the peak shifted to 190 kDa (Figure 4B), consistent with the formation of Rad1/Rad10 complexes with 1:1 stoichiometry. In support of this conclusion, the coelution of Rad1 (Figure 3C) and Rad10 (Figure 3D) in the 190-kDa peak was confirmed by immunoblotting. As anticipated, we detected a second peak (Figure 3B) that eluted with the expected relative molecular mass of homogeneous Rad10 protein. This peak was approximately 40% the area of the peak observed when the same quantity of Rad10 alone was analyzed by gel filtration in the absence of Rad1 protein (data not shown). From these results, we estimate that >90% of the Rad1 molecules formed stable 1:1 complexes with Rad10 molecules.

Rad1/Rad10 Complexes Have DNA Endonuclease Activity. In addition to its ability to complex with purified Rad10 protein, purified Rad1 protein supports the endonucleolytic degradation of single-stranded DNA (Tomkinson et al., 1993) and the nicking of supercoiled duplex molecules (see later), when complexed with Rad10 protein. When fractions from the more rapidly eluting gel filtration peak (Figure 3B) were incubated with supercoiled PM2 DNA, we observed conversion of the supercoiled material to the nicked form, indicative of endonuclease activity (data not shown, see Figure 5A for analogous data). No endonuclease activity was observed in similar assays with fractions containing Rad1 protein alone (data not shown). Hence, Rad1 protein purified by the procedure described here is also able to support endonuclease activity.

Polynucleotide Substrate Specificity of the Rad1/Rad10 Endonuclease. We previously reported that the Rad1/Rad10 endonuclease degrades single-stranded circular DNA by an endonucleolytic mechanism (Tomkinson et al., 1993). In Figure 4, we show the degradation of ^{3}H-labeled fd phage DNA as a function of the concentration of Rad1/Rad10 endonuclease. The products of this reaction were analyzed by agarose gel electrohoresis (Figure 4A) and by sedimentation in alkali sucrose gradients (Figure 4B). As reported previously, no degradation of the DNA was detected by either Rad1 or Rad10 protein alone (Figure 4A). Under conditions that approached complete digestion, oligonucleotides ranging from 100 to 500 nucleotides in length were produced. Assuming an average oligonucleotide size of 300 bases, we estimate that the overall rate of cleavage was 0.05 nick complex^{-1} min^{-1}. The absence of significant amounts of radioactivity at the top of the gradients indicates that there is no detectable exonuclease activity associated with the Rad1/Rad10 complex. Consistent with this conclusion, incubation of the enzyme with ^{3}H-labeled native or heat-denatured *E. coli* chromosomal DNA did not result in the formation of acid-soluble radioactivity (data not shown).

Single-stranded DNA endonucleases are able to nick supercoiled DNA because of the partial single-stranded character of such molecules (Kato et al., 1973). The Rad1/Rad10 endonuclease nicked positively supercoiled PM2 DNA, whereas neither Rad1 nor Rad10 protein alone had nuclease

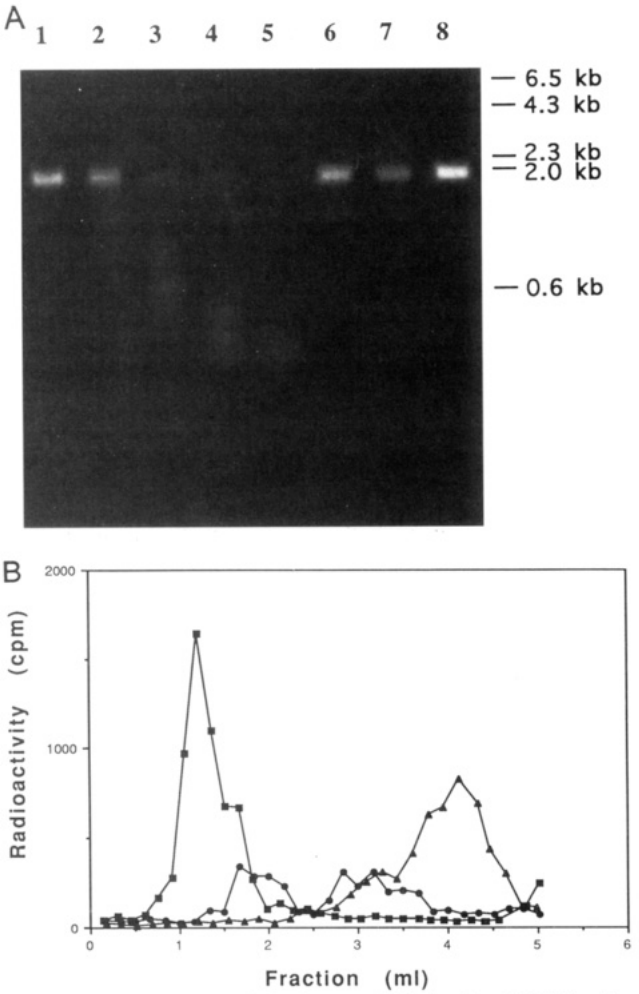

FIGURE 4: Degradation of single-stranded circular DNA by the Rad1/Rad10 complex. (A) Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, and 81 ng of [^{3}H]DNA from bacteriophage fd were incubated for 15 min at 37 °C with the following: lane 1, 0.131 pmol of Rad1 protein and 0.122 pmol of Rad10 protein; lane 2, 0.262 pmol of Rad1 protein and 0.244 pmol of Rad10 protein; lane 3, 0.525 pmol of Rad1 protein and 0.488 pmol of Rad10 protein; lane 4, 1.05 pmol of Rad1 protein and 0.975 pmol of Rad10 protein; lane 5, 2.1 pmol of Rad1 protein and 1.95 pmol of Rad10 protein; lane 6, 1.95 pmol of Rad10 protein; lane 7, 2.1 pmol of Rad1 protein; lane 8, no addition. After incubation, samples were heated at 90 °C for 2 min and electrophoresed through a 0.8% agarose gel. DNA was detected by staining with ethidium bromide. The positions of molecular mass standards (λ-*Hin*dIII digest, Pharmacia) are indicated on the right. (B) Similar reactions containing 405 ng of [^{3}H]DNA from bacteriophage fd were carried out in a final volume of 50 μL. These reactions contained no addition (■), 2.9 pmol of Rad10 protein and 3.15 pmol of Rad1 protein (●), and 5.8 pmol of Rad10 protein and 6.3 pmol of Rad1 protein (▲). Reactions were terminated by the addition of 5 μL of 0.5 M EDTA and then analyzed by alkaline sucrose gradient sedimentation as described under Experimental Procedures. The position of a molecular mass standard [poly(dA) (~540 nucleotides, Pharmacia)], sedimented in a separate gradient under identical conditions, is indicated.

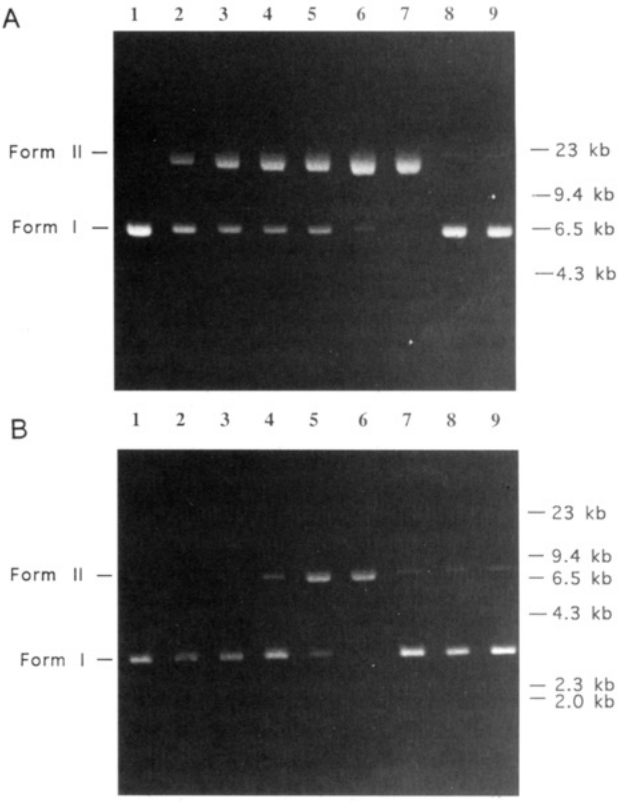

FIGURE 5: Endonucleolytic cleavage of positively and negatively supercoiled DNA by the Rad1/Rad10 complex. (A) Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, and 120 ng of bacteriophage PM2 DNA were incubated for 15 min at 37 °C with the following: lane 1, no addition; lane 2, 0.066 pmol of Rad1/Rad10 complex; lane 3, 0.131 pmol of Rad1/Rad10 complex; lane 4, 0.203 pmol of Rad1/Rad10 complex; lane 5, 0.525 pmol of Rad1/Rad10 complex; lane 6, 1.05 pmol of Rad1/Rad10 complex; lane 7, 2.1 pmol of Rad1/Rad10 complex; lane 8, 3.9 pmol of Rad10 protein alone; lane 9, 2.1 pmol of Rad1 protein alone. After incubation, samples were placed on ice to stop the reaction and then separated through a 0.8% agarose gel containing ethidium bromide. The positions of molecular mass standards (λ-*Hin*dIII digest, Pharmacia) are indicated on the right, and the positions of supercoiled (form I) and nicked (form II) PM2 DNA are indicated on the left. The quantity of the Rad1/Rad10 complex assumes complete efficiency of complex formation. These reactions contained a 1.8 molar excess of Rad10. Similar results were obtained with equimolar mixtures of these proteins. (B) Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, and 120 ng of bacteriophage ϕX174 RF form I DNA were incubated for 15 min at 37 °C with the following: lane 1, 0.066 pmol of Rad1/Rad10 complex; lane 2, 0.131 pmol of Rad1/Rad10 complex; lane 3, 0.203 pmol of Rad1/Rad10 complex; lane 4, 0.525 pmol of Rad1/Rad10 complex; lane 5, 1.05 pmol of Rad1/Rad10 complex; lane 6, 2.1 pmol of Rad1/Rad10 complex; lane 7, 3.9 pmol of Rad10 protein alone; lane 8, 2.1 pmol of Rad1 protein alone, and lane 9, no addition. After incubation, the samples were placed on ice to stop the reaction and then separated through a 0.8% agarose gel containing ethidium bromide. The positions of molecular mass standards (λ-*Hin*dIII digest, Pharmacia) are indicated on the right, and the positions of the supercoiled (form I) and nicked (form II) ϕX174 are indicated on the left.

activity against this substrate (Figure 5A). We obtained similar results with negatively supercoiled ϕX174 RF DNA (Figure 5B). The rate of conversion of supercoiled form I DNA to nicked form II DNA was quantitated by scanning densitometry of a photographic negative of the ethidium bromide stained gel and was found to be proportional to the amount of Rad1/Rad10 complex added. Assuming 1 nick per DNA molecule, we estimate the rate of cleavage to be 0.01 nick complex^{-1} min^{-1}. Although the Rad1/Rad10 endonuclease is less active on supercoiled DNA than single-stranded DNA, it is easier to detect and quantitate form II DNA generated by nicking of supercoiled DNA than the heterogeneous population of oligonucleotides generated by degradation of single-stranded DNA circles, which manifest as a smear in electrophoretic gels. Irradiation of supercoiled PM2 DNA with 400 J/m^2 of 254-nm UV light prior to incubation with the Rad1/Rad10 complex neither detectably enhanced nor inhibited the endonuclease activity (data not shown). Not surprisingly, preincubation of negatively or positively supercoiled DNA with DNA topoisomerase I to relax the extent of supercoiling reduced the extent of cleavage by the Rad1/Rad10 endonuclease by ~5-fold (data not shown).

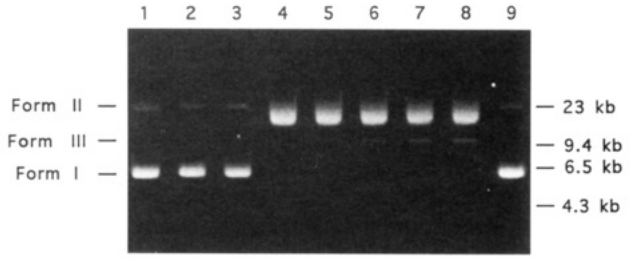

FIGURE 6: Time course of degradation of supercoiled PM2 DNA. Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, and 120 ng of PM2 DNA were incubated at 37 °C for 40 min with the following: lane 1, 0.7 pmol of Rad1 protein; lane 2, 1.0 pmol of Rad10 protein; and lane 9, no addition. Aliquots (20 μL) were taken from a reaction mixture (120 μL) containing 50 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, 720 ng of PM2 DNA, and 4.2 pmol of Rad1/Rad10 complex after incubation at 37 °C for the following: lane 3, 0 min; lane 4, 5 min; lane 5, 10 min; lane 6, 20 min; lane 7, 30 min; and lane 8, 40 min. Reactions were terminated by placing on ice, and then samples were electrophoresed through a 0.8% agarose gel containing ethidium bromide. The positions of molecular mass standards (λ-*Hin*dIII digest, Pharmacia) are indicated on the right, and the positions of the supercoiled (form I), nicked (form II), and linear (form III) PM2 DNAs are indicated on the left.

At high Rad1/Rad10 concentrations, a small amount of linear DNA PM2 was observed (Figure 6). Examination of the kinetics of product formation demonstrated that the conversion of supercoiled (form I) to nicked (form II) molecules is a relatively rapid reaction compared to the slow accumulation of linear (form III) molecules. This suggests that following the rapid nicking of supercoiled molecules, the Rad1/Rad10 endonuclease cleaves transient single-stranded regions opposite nicks at a significantly reduced rate. The Rad1/Rad10 endonuclease did not degrade linear duplex substrates such as *E. coli* DNA (measured by the release of acid-soluble radioactivity) or full-length linear PM2 DNA (measured by agarose gel electrophoresis).

DISCUSSION

We previously reported that a complex of Rad1 and Rad10 proteins degrades single-stranded DNA by an endonucleolytic mechanism (Tomkinson et al., 1993). In the present study, we have described the purification of Rad1 protein to apparent physical homogeneity. Additionally, we have developed an improved purification scheme for Rad10 protein, and have further characterized the physical and catalytic properties of the Rad1/Rad10 endonuclease complex.

The observation that stable Rad1/Rad10 complexes are generated in the absence of DNA both *in vitro* (Bailly et al., 1992; Bardwell et al., 1992) and *in vivo* (Bardwell et al., 1993) suggests that such complexes are constitutively present in cells. Studies on the level of expression of the *RAD1* and *RAD10* genes in untransformed yeast cells suggest that Rad10 protein is present in molar excess (Bardwell et al., 1990; Weiss & Friedberg, 1985; Yang, 1987). Hence, excess Rad10 protein that is synthesized following the transformation of cells with expression plasmids is not expected to complex with constitutive Rad1 protein, and is potentially available for purification as a single polypeptide. In contrast, the purification of free Rad1 protein would presumably require its expression at levels significantly greater than the constitutive level of free Rad10 protein in cells. We overcame this potential problem by overexpressing Rad1 protein in a yeast strain deleted of the *Rad10* gene.

The predicted molecular weight of Rad1 protein is 126 200 (Reynolds et al., 1987). The protein exhibits anomalous behavior in several different techniques used for estimating relative molecular mass. During denaturing gel electrophoresis, *in vitro*-translated Rad1 protein (Bardwell et al., 1992) and that overexpressed in either yeast or *E. coli* have a relative molecular mass of 150 kDa. Hence, this discrepancy is probably not the result of posttranslational modification and may reflect the acidic nature of this protein (Reynolds et al., 1987). In addition, Rad1 protein elutes during gel filtration with a relative molecular mass of 160 kDa, suggesting that this protein also has an asymmetric shape.

Complex formation between Rad1 and Rad10 proteins *in vitro* was previously investigated by immunoprecipitation (Bailly et al., 1992; Bardwell et al., 1992, 1993) and by the binding of Rad1 to Rad10 protein convalently attached to an inert matrix (Tomkinson et al., 1993). These experiments did not directly address the stoichiometry of complex formation. We have now shown that the Rad1/Rad10 complex has a relative molecular mass of 190 kDa determined by gel filtration. This value is almost precisely that expected from a complex of one molecule of Rad1 protein (relative molecular mass = 160 kDa) and one molecule of Rad10 protein (relative molecular mass = 25 kDa). Futhermore, the Rad1/Rad10 complex purified by gel filtration is active as an endonuclease, confirming that the two proteins function as subunits of this endonuclease.

The following properties of the Rad1/Rad10 complex indicate that it is a single-strand-specific endonuclease: (i) the enzyme degrades single-stranded DNA circles by an endonucleolytic mechanism; (ii) the enzyme nicks supercoiled DNA; (iii) relaxation of the supercoiling by topoisomerase I reduces the endonuclease activity; (iv) the enzyme does not cleave linear duplex molecules; (v) the enzyme does not possess exonuclease activity.

The specific activity of the Rad1/Rad10 endonuclease is low compared with other single-strand-specific endonucleases, such as that purified from mammalian mitochonidria (Tomkinson & Linn, 1986), and this enzyme does not preferentially cleave UV-irradiated DNA. These observations may reflect the fact that *in vivo* the enzyme reacts with a particular DNA structure generated by the action of other proteins, and/or the specificity and reactivity of the enzyme are modulated by other protein–protein interactions. During NER in *E. coli*, UvrC protein in isolation has a weak endonuclease activity with no specificity for DNA damage. However, in combination with UvrA and UvrB proteins, DNA damage-specific incisions occur at a significantly higher rate (Caron & Grossman, 1988). Alternatively, one of the purified Rad proteins may be inactive. This is a relevant concern since both proteins were purified by nonfunctional immunoblotting assays. However, independent experiments have demonstrated that both purified Rad1 and Rad10 proteins restore DNA damage-dependent repair synthesis in cell-free extracts prepared from the respective *rad1* and *rad10* mutant strains (Wang et al., 1993). The question of whether the specific activity of the *in vitro*-constituted Rad1/Rad10 endonuclease accurately reflects the activity of the *in vivo* complex can be definitively addressed by overexpressing both proteins in the same cell and then isolating and characterizing the complex.

Of the multiple yeast genes required for the recognition of base damage and specific incision of DNA at or near such lesions, the *RAD1* and *RAD10* genes are unique in that they are also involved in a specialized mitotic intrachromosomal recombination pathway (Friedberg et al., 1991). A comparison of the recombination intermediates generated in wild-type and *rad1* mutant strains led to the suggestion that the product of the *RAD1* gene removes 3′ nonhomologous DNA single strands by an endonucleolytic mechanism (Fishman-Lobell & Haber, 1992). Hence, the endonuclease activity associated with the Rad1/Rad10 complex is consistent with the predicted roles of these proteins in mitotic recombination.

In the *E. coli* NER pathway, the DNA incisions on either side of sites of base damage are catalyzed by different proteins in a nonconcerted reaction. The 3′ incision is catalyzed by UvrB protein at the fifth phosphodiester bond 3′ to the site of base damage, following which the 5′ incision is catalyzed by UvrC protein at the eighth phosphodiester bond 5′ to the site of base damage. These reactions generate an oligonucleotide 13 nucleotides in length that is excised from the DNA (Lin et al., 1992; Lin & Sancar, 1992). NER supported by human and *Xenopus laevis* cell-free extracts is associated with the generation of excision products that are 27–32 nucleotides in length, with the 3′ incision at the 5th phosphodiester bond 3′ to the lesion and the 5′ incision at the 22–24th phosphodiester bond 5′ to the lesion (Huang et al., 1992; Svodoba et al., 1993). The apparent evolutionary conservation of many yeast and human NER genes suggests that the bimodal incision process observed in human cell-free extracts is likely conserved in yeast (Friedberg, 1992).

Since the Rad1/Rad10 complex in isolation is a weak endonuclease with no apparent DNA damage specificity, we predict that this enzyme is targeted to DNA lesions by specific protein–protein interactions that facilitate its role in the process of DNA incision during NER. Assuming that in *S. cerevisiae* damage-specific incision is first catalyzed 3′ to the site of DNA damage (as is apparently the case in *E. coli*) by an endonuclease other than the Rad1/Rad10 complex, the displacement of a single-stranded tail containing the base damage (perhaps by a DNA helicase) could generate a 3′ single-stranded structure resembling the recombinational intermediate discussed above (Fishman-Lobell & Haber, 1992). Cleavage of this structure by the Rad1/Rad10 endonuclease would then generate the 5′ nick. Such an unconcerted reaction mode predicts that damage-specific DNA strand breaks should be detectable in *rad1* or *rad10* mutants. However, previous studies have shown that mutant strains that are also defective in DNA ligase activity do not accumulate detectable single-strand breaks *in vivo* after exposure to UV radiation (Wilcox & Prakash, 1981). Hence, it is possible that the Rad1/Rad10 endonuclease catalyzes both the 3′ and 5′ incisions. Alternatively, the incisions may be made by two different nucleases (one of which is the Rad1/Rad10 complex) in a reaction mechanism that requires both to be present at the 3′ and 5′ incision sites before cleavage can occur.

The purification of Rad1 and Rad10 proteins and the characterization of the Rad1/Rad10 endonuclease will permit the use of this enzyme as a reagent in further studies on the molecular mechanisms of intrachromosomal mitotic recombination and NER in eukaryotes. The homology between repair genes from *S. cerevisiae* and humans in general, and that between the *S. cerevisiae RAD10* and human *ERCC1* genes in particular (van Duin et al., 1986), suggests that there is a human enzyme that is functionally homologous with the Rad1/Rad10 endonuclease. We anticipate that this enzyme consists of Ercc1 protein complexed to a protein that is encoded by an as yet unidentified human gene homologous to the *S. cerevisiae RAD1* gene. The recent observations that Ercc1 protein specifically associates with other mammalian NER proteins (Biggerstaff et al., 1993; van Vuuren et al., 1993) are consistent with this model.

ACKNOWLEDGMENT

We thank Doug Johnston and Christina Wooton for assistance with yeast fermentation and our laboratory colleagues for discussions and for critical review of the manuscript.

REFERENCES

Aguilera, A., & Klein, H. L. (1989) *Genetics 123*, 683–694.
Bailly, V., Sommers, S. H., Sung, P., Prakash, L., & Prakash, S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8273–8277.
Bardwell, L., Burtscher, H., Weiss, W. A., Nicolet, C. M., & Friedberg, E. C. (1990) *Biochemistry 29*, 3119–3126.
Bardwell, L., Bardwell, A. J., & Friedberg, E. C. (1992) *Mol. Cell. Biol. 12*, 3041–3049.
Bardwell, A. J., Bardwell, L., Johnson, D. K., & Friedberg, E. C. (1993) *Mol. Microbiol. 8*, 1177–1188.
Biggerstaff, M., Szymkowski, D. E., & Wood, R. D. (1993) *EMBO J. 12*, 3685–3692.
Bradford, M. M. (1976) *Anal. Biochem. 76*, 248–254.
Caron, P. R., & Grossman L. (1988) *Nucleic Acids Res. 16*, 7855–7865.
Espejo, R. T., & Canelo, E. S. (1968) *Virology 34*, 738–747.
Fishman-Lobell, J., & Haber, J. (1992) *Science 258*, 480–484.
Formosa, T., Barry, J., Alberts, G. M., & Greenblatt, J. (1991) *Methods Enzymol. 208*, 24–54.
Friedberg, E. C. (1992) *Cell 71*, 887–889.
Friedberg, E. C., Siede, W., & Cooper, A. J. (1991) in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis and Energetics* (Broach, J., Jones, E., & Pringle, J., Eds.) pp 147–192, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Goldmark, P. J., & Linn, S. (1970) *Proc. Natl. Acad. Sci. U.S.A. 73*, 434–441.
Higgins, D. R., Prakash, S., Reynolds, P., & Prakash, L. (1983) *Gene 26*, 119–126.
Huang, J.-C., Svodoba, D. L., Reardon, J. T., & Sancar, A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3664–3668.
Kato, A. C., Bartok, K., Fraser, M. J., & Denhardt, D. T. (1973) *Biochim. Biophys. Acta 308*, 66–78.
Kuhnlein, U., Penhoet, E. E., & Linn, S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1169–1173.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lehman, I. R. (1960) *J. Biol. Chem. 235*, 1479–1487.
Lin, J.-J., & Sancar, A. (1992) *J. Biol. Chem. 267*, 17688–17692.
Lin, J.-J., Phillips, A. M., Hearst, J. E., & Sancar, A. (1992) *J. Biol. Chem. 267*, 17693–17700.
Prakash, L. (1977) *Mol. Gen. Genet. 153*, 125–128.
Reynolds, R., & Friedberg, E. C. (1981) *J. Bacteriol. 146*, 692–704.
Reynolds, P., Prakash, L., Dumais, D., Perozzi, G., & Prakash, S. (1985) *EMBO J. 4*, 3548–3552.
Reynolds, P., Prakash, L., & Prakash, S. (1987) *Mol. Cell. Biol. 7*, 1012–1021.
Schiestl, R. H., & Prakash, S. (1989) *Mol. Cell. Biol. 8*, 3619–3626.
Schiestl, R. H., & Prakash, S. (1990) *Mol. Cell. Biol. 10*, 2485–2491.
Sung, P., Prakash, L., & Prakash, S. (1992) *Nature 355*, 743–745.
Svodoba, D. L., Taylor, J.-S., Hearst, J. E., & Sancar, A. (1993) *J. Biol. Chem. 268*, 1931–1936.
Thomas, B. J., & Rothstein, R. (1989) *Genetics 123*, 725–738.
Tomkinson, A. E., & Linn, S. (1986) *Nucleic Acids Res. 12*, 9579–9593.
Tomkinson, A. E., Tappe, N. J., & Friedberg, E. C. (1992) *Biochemistry 31*, 11762–11771.
Tomkinson, A. E., Bardwell, A. J., Bardwell, L., Tappe, N. J., & Friedberg, E. C. (1993) *Nature 362*, 860–862.
Unrau, P., Whatcroft, R., & Cox, B. S. (1971) *Mol. Gen. Genet. 354*, 8359–8362.
van Duin, M., de Wit, J., Odijk, H., Westerveld, A., Yasui, A., Koken, M. H. M., Hoeijmakers, J. H. J., & Bootsma, D. (1986) *Cell 44*, 913–923.
van Vuuren, A. J., Appeldoorn, E., Odijk, H., Yasui, A., Jaspers, N. G. J., Bootsma, D., & Hoejmakers, J. H. J. (1993) *EMBO J. 12*, 3693–3701.
Wang, Z., Wu, X., & Friedberg, E. C. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 4907–4911.
Weiss, W. A., & Friedberg, E. C. (1985) *EMBO J. 4*, 1575–1582.
Wilcox, D. R., & Prakash, L. (1981) *J. Bacteriol. 148*, 618–623.
Yang, E. (1987) Ph.D. Thesis, Stanford University.