Saccharomyces cerevisiae Possesses Two Functional Homologues of *Escherichia coli* Endonuclease III[†]

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ABSTRACT: We previously identified two distinct genes of *Saccharomyces cerevisiae* redoxyendonuclease (*SCR1* and *SCR2*) which possess a high degree of sequence similarity to *Escherichia coli* endonuclease III [Augeri, L., Lee, Y. M., Barton, A. B., and Doetsch, P. W. (1997) *Biochemistry 36*, 721−729]. The proteins encoded by *SCR1* and *SCR2* were overexpressed in *E. coli* and purified to apparent homogeneity. Both proteins recognized and cleaved DNA substrates containing dihydrouracil, 2,6-diamino-4-hydroxy-5*N*-methylformamidopyrimidine (FaPy-7-MeGua), and abasic sites but not DNA substrates containing uracil or 8-oxoguanine. Purified Scr2, but not Scr1, possesses spectral properties which indicate the presence of an iron−sulfur center. Kinetic parameters for Scr1 and Scr2 were determined by using an oligonucleotide containing a single dihydrouracil. Analysis of the deduced amino acid sequences of Scr1 and Scr2 suggests that Scr2 bears an iron−sulfur motif, while Scr1 does not have this motif. However, Scr1 has a long, positively charged N-terminus that could be a mitochondrial transit sequence. Targeted gene disruption of *SCR1* and *SCR2* produced a double mutant that had no detectable enzymatic activity against the dihydrouracil-containing substrate. Northern blot analysis showed that *SCR1* was induced by menadione, but *SCR2* was not. These results indicate that although Scr1 and Scr2 are both functional homologues of *E. coli* endonuclease III, they differ from each other with respect to their amino acid sequences and inducibility by DNA damaging agents, suggesting that their precise biological roles may be different.

Reactive oxygen species (ROS)[1] generated in cells either as byproducts of aerobic metabolism or as a consequence of exposure to ionizing radiation and other oxidizing agents can cause damage to DNA bases and strand breaks (*1*, *2*). Unrepaired oxidative damage to DNA has been suggested to play a role in cancer, aging, and other degenerative processes (*3*, *4*). In most organisms, the repair of oxidative DNA base damage is thought to be primarily mediated via the base excision repair (BER) pathway (*5*).

Escherichia coli endonuclease III has been shown to remove base damage by a DNA N-glycosylase activity and then to cleave the phosphodiester backbone at the resulting apurinic/apyrimidinic (AP) site via a β-elimination reaction (*6*). Endonuclease III is thought to recognize and process a wide variety of pyrimidine base damages including ring saturation, ring fragmentation, and ring contraction products (*5*). Functional homologues of endonuclease III have been identified in a number of prokaryotes (*7*, *8*) and eukaryotes, including humans (*9*, *10*), indicating the highly conserved nature of this DNA repair pathway. In general, endonuclease III homologues possess two highly conserved regions, a helix−hairpin−helix motif corresponding to the putative active site and a $C-X_6-C-X_2-C-X_5-C$ segment containing an iron−sulfur center near the C-terminus (*11*). We and others have previously shown that *Saccharomyces cerevisiae* possesses endonuclease III-like activity and have termed the enzyme redoxyendonuclease (*12*−*14*). We also have previously reported that *S. cerevisiae* possesses two genes, *SCR1* and *SCR2* (also called *NTG1* and *NTG2*), encoded on chromosomes I and XV, respectively (*14*). Both genes possess a high degree of amino acid sequence similarity to endonuclease III and are also closely related (41% identity, 63% similarity) to each other (*9*). Wild-type yeast strains contain two chromatographically separable endonuclease III-like activities, and *scr1* mutants contain only one of these activities (*14*). Here, we have overexpressed and purified the Scr1 and Scr2 proteins to apparent homogeneity and present evidence that Scr2, but not Scr1, contains an iron−sulfur center. We have examined the activities of purified

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[1] Abbrevations: AP, apurinic or apyrimidinic; BER, base excision repair; DEPC, diethyl pyrocarbonate; DHU, 5,6-dihydrouracil; FPLC, fast-protein liquid chromatography; IPTG, isopropyl β-D-thiogalactoside; FaPy-Gua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; Fapy-7-MeGua, 2,6-diamino-4-hydroxy-5*N*-methylformamidopyrimidine; Fpg, formamidopyrimidine-DNA glycosylase; 8-oxoG, 8-oxoguanine; PCIA, phenol−chloroform−isoamyl alcohol; ROS, reactive oxygen species; Scr, *Saccharomyces cerevisiae* redoxyendonuclease; SD-Leu, synthetic dextrose lacking leucine medium; SDS, sodium dodecyl sulfate; SSC, sodium chloride sodium citrate; RT−PCR, reverse transcriptase−polymerase chain reaction; U, uracil; UDG, uracil-DNA glycosylase; YPD, yeast extract peptone dextrose.

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Scr1 and Scr2 as well as extracts from Scr1 and Scr2 mutants on DNA substrates containing various defined base damage products. In addition, the inducibility of *SCR1* and *SCR2* in response to oxidative stress was investigated. Our results demonstrate that *S. cerevisiae* possesses two functional homologues of *E. coli* endonuclease III and is the first such example reported to date.

MATERIALS AND METHODS

Strains and Vectors. The *E. coli* strain DH5α was used for subcloning and propagation of plasmids, and BL21 (DE3) was used for protein expression. *S. cerevisiae* haploid strains used were *SJR751* (*MAT*α *ade2-101 his3*Δ*200 leu2-R ura3*Δ*Nco lys2*Δ*Bgl), SJR832 (MAT*α *ade2*−*101 his3*Δ*200 leu2-R ura3*Δ*Nco lys2*Δ*Bgl scr1::LEU2), SJR834 (MAT*α *ade2-101 his3*Δ*200 leu2-R ura3*Δ*Nco lys2*Δ*Bgl scr1::LEU2 scr2::hisG-URA3-hisG), SJR835 (ade2-101 his3*Δ*200 leu2-R ura3*Δ*Nco lys2*Δ*Bgl scr2::hisG-URA3-hisG*). pGEX-2T was obtained from Pharmacia, and pRSETA was obtained from Invitrogen.

Mutant Strain Construction. The plasmid pLF298 containing a portion of *S. cerevisiae* chromosome I with *scr1*::*LEU2* was a gift from Dr. Arnold Barton (UMDNJ, Newark) and was generated as described previously (*15*). pLF298 digested with *Nco*I and *Nde*I was transformed into SJR751 using a lithium acetate protocol (*16*). Transformants were selected on SD-Leu medium. The disruptions were confirmed by PCR using primers for the 5′and 3′ ends of *SCR1* generating a 1.4-kb product for wild-type or a 3.2-kb product for *scr1::LEU2*.

SCR2 was cloned by PCR using the following primers: 5′-GGCTCGAGAGAGAGGAAAGTAGG-3′ and 5′-GCAGCTGCTATTTTTTCTT GTGTC-3′. The gene was inserted into the pGEM-7Zf vector (Promega), and the hisG-*URA3*-hisG cassette (*17*) was inserted into the *Eco*RI site of *SCR2*. The resulting plasmid was digested with *Xho*I and *Sac*I and transformed into SJR751 or SJR832. Transformants were selected on SD-ura medium. Disruptions were confirmed by PCR using internal primers flanking the *Eco*RI site of *SCR2* (5′-GGACAATTTCGATTCAGATATACCG-3′,5′-GGGTTAAGGTGTAAAATGT GTCGG-3′), and an internal primer of the hisG sequence of *S. typhimurium* (5′-GCGTTTGAGGAGGTGCGGATATGAGG-3′) producing a 0.2 kb product for wild-type or a 0.5 kb product for *scr2::hisG-URA3-hisG*.

Oligonucleotide Substrates Containing DNA Base Damage. The dihyrodeoxyuridine dimethoxytrityl-blocked phosphoramidite building block was synthesized by Glen Research (Sterling, VA). Dihydrouracil (DHU)-containing oligomer (DHU-37mer) with the sequence 5′-CTT-GGA-CTG-GAT-GTC-GGC-AC**X**-AGC-GGA-TAC-AGG-AGC-A-3′ (X = DHU) and its complementary strand (G is opposite DHU) were synthesized by Research Genetics (Birmingham, AL). Undamaged 37mer (UND-37mer, X = C) and 8-oxoguanine-containing 37mer (8-oxoG-37mer, X = 8-oxoG) were synthesized by National Bioscience Incorporated, and uracil-containing 37mer (U-37mer, X = uracil) was synthesized by the Emory University Microchemical Facility. Oligonucleotides were 3′-end-labeled using terminal transferase and [α-^{32}P]ddATP (Amersham, 3000 Ci/mmol) (*18*). End-labeled single-stranded DNA was gel-purified on 20% denaturing polyacrylamide gels. End-labeled duplex oligonucleotides were gel-purified on a 20% nondenaturing polyacrylamide gel (*14*). DNA was resuspended in either TE buffer or 10 mM HEPES−KOH, pH 8.0, and 2 mM EDTA (HE) and stored at −20 °C. End-labeled duplex URA-37mer (20−50 pmol) was incubated with uracil-DNA glycosylase (6 units) for 30 min at 30 °C in UDG buffer (30 mM HEPES−KOH, pH 7.5, 1 mM EDTA, and 50 mM NaCl) to generate the abasic site containing oligonucleotide, AP-37mer. AP-37mer was extracted with PCIA (phenol−chloroform−isoamyl alcohol, 29:19:1, v/v/v, equilibrated with HE buffer and 0.1% 8-hydroxyquinoline) and was evaluated for its AP site content by cleavage with 0.1 M piperidine at 90 °C for 20 min. Reaction products were electrophoresed on 20% denaturing (7 M urea) polyacrylamide gels (DNA sequencing gels), which were subjected to autoradiography. DNA strand scission product formation was determined by phosphorimager analysis of DNA sequencing gels (Molecular Dynamics model 445 SI).

Enzymes and Chemicals. Escherichia coli endonuclease III was a gift from Dr. Richard Cunningham (SUNY, Albany). *Escherichia coli* formamidopyrimidine-DNA glycosylase (Fpg) and endonuclease IV were gifts from Dr. Yoke Wah Kow (Emory University). T4 polynucleotide kinase was obtained from New England Biolabs. Restriction enzymes were obtained from Promega. Terminal transferase was from Promega, and *Taq* DNA polymerase for PCR experiments was from Fisher. Menadione was from Sigma. All other chemicals were of the highest grade commercially available.

Enzyme Assay. A 50-μL reaction mixture containing buffer B (15 mM KH_2PO_4, pH 6.8, 10 mM EDTA, and 10 mM β-mercaptoethanol) plus 40 mM KCl and end-labeled duplex oligonucleotide was incubated for 30 min at 37 °C with purified Scr1 (0.1−10 ng), Scr2 (0.1−10 ng), or endonuclease III (1 ng). DNA was extracted with PCIA and ethanol-precipitated, and the reaction products were analyzed on DNA sequencing gels as previously described (*14*).

Generation of FaPy-7-MeGua in Supercoiled DNA and DNA Nicking Assay. One microgram of pRSETA supercoiled DNA was dissolved in 100 μL of TE buffer. The supercoiled DNA was incubated with 4 mM DMS for 10 min at 37 °C, ethanol precipitated, and resuspended in 100 μL of TE buffer. To create FaPy-7-MeGua residues in the supercoiled DNA, 100 μL of DNA was incubated with 0.2 M NaOH for 20 min at 37 °C. Following treatment, the pH of the solution was adjusted to 8.0 with 1 M Tris-HCl buffer, and the DNA was ethanol precipitated and resuspended in TE buffer (*19*). The supercoiled DNA was incubated with 40 ng of endonuclease IV at 37 °C for 20 min to remove AP sites. The nicked plasmid was incubated with Scr1, Scr2, *E. coli* endonuclease III, or Fpg at 37 °C for 30 min. Following incubation, the reaction was terminated by the addition of 3 μL of stop solution (10% SDS, 0.25% bromophenol blue, and 25% Ficoll), loaded onto a 0.9% agarose minigel, and electrophoresed for 90 min at 90 V. Following electrophoresis, the gel was visualized by staining with ethidium bromide and exposure to UV light.

Overexpression and Purification of Scr1 and Scr2 Proteins. The *SCR1* gene coding region (1200 bp) was amplified by PCR from genomic yeast DNA using primers 5′-GGTCCATGCAAAAGGTC-3′ and 5′-GGAATTCTAGTC-

CTCTACT TTAA-3′, and the *SCR2* gene coding region (1120 bp) was amplified by PCR from genomic yeast DNA using primers 5′-GGCTCGAGATGAGAGAGAAAGT A-3′ and 5′-GGCAGCTGCTATTTTTTCTTGTGTC-3′. The amplified *SCR1* gene coding fragments were cloned into *Bam*HI and *Eco*RI restriction sites of pRSETA, and the amplified *SCR2* gene coding fragments were cloned into *Xho*I and *Pvu*II restriction sites of pGEXT-2T. In the pRSETA vector the coding region is expressed in-frame with a GST leader sequence from the bacteriophage T7 promoter. In the pGEX-2T vector the coding region is expressed in-frame with a hexahistidine (His_6) leader sequence from the bacteriophage T7 promoter. Expression of Scr1 protein was achieved by induction for 2 h at room temperature by the addition of 0.1 mM isopropyl β-D-thiogalactoside (IPTG). Cells were lysed by sonication, and the 12000*g* supernatant was applied to glutathione−Sepharose affinity beads (*20*). Following displacement from the affinity beads, the Scr1 fraction was further purified by Mono S FPLC. Expression of Scr2 protein was achieved by IPTG induction for 2 h at room temperature in the presence of 1 M sorbitol (final concentration) and 2.5 mM betaine (final concentration) to increase protein solubility (*21*). After cell lysis by sonication, the 12000*g* supernatant was fractionated by Mono S FPLC. The Scr2 protein was eluted with a 0.05−1 M NaCl gradient. Fractions that contained Scr2 protein were loaded onto a Ni^+-charged Hi-Trap column (Pharmacia). The column was washed, and bound proteins were eluted with 0.3 M imidazole buffer (pH 7.4) and were fractionated again by Mono S FPLC.

Kinetic Assays. Enzyme reaction mixtures (50 μL) containing various amounts of ^{32}P-labeled substrates (50−200 nM) were incubated with a final concentration of 20 nM Scr1 or 60 nM Scr2 for 0−5 min at 37 °C. The samples were electrophoresed on DNA sequencing gels, and the bands corresponding to uncleaved and cleaved DNA species were quantitated by phosphorimager analysis. Data from a single reaction time point (1 min) were analyzed using Microcal Origin statistical analysis software (Microcal Software). Reaction rates were calculated as nanomoles of product (cleaved oligonucleotide substrate) formed per minute per nanogram of protein. The apparent K_m, V_{max}, and turnover number (K_{cat}) values were determined from Lineweaver−Burk plots of averaged data (±standard deviation) from four experiments.

Northern Blot Analysis. Yeast cells (10 mL) grown in YPD (1% yeast extract, 2% peptone, and 2% dextrose) to an OD_{600} of 2.0 were exposed to 0.3 mM (final concentration) menadione for 2 h, or 1.5 mM hydrogen peroxide (final concentration) for 30 min, at 30 °C. RNA was isolated using the hot phenol method (*22*). The cells were harvested by centrifugation and resuspended in 400 mL of 50 mM sodium acetate, pH 5.3, and 10 mM EDTA. The resuspended cells were lysed with 10% SDS. The suspension was vortexed, and an equal volume of fresh phenol was added. The mixture was incubated at 65 °C for 4 min and then centrifuged for 2 min at maximum speed. Sodium acetate (3 M, pH 5.2) and ethanol were added to the aqueous phase to precipitate the RNA. After an with 80% ethanol wash, the pellet was dried, resuspended in DEPC-treated water, and stored at −80 °C until use. Total RNA (20 μg) isolated from yeast which were either untreated, exposed to hydrogen peroxide, or exposed to menadione were heated at 65 °C for 10 min and loaded onto a 1% agarose−formaldehyde gel. The gel was rinsed for 20 min in 10× SSC, and RNA was transferred to a nylon membrane (MSI) by the downward capillary method (*23*). Following transfer, the membrane was rinsed in 2× SSC for 5 min and baked at 80 °C for 1 h. Before hybridization with the cDNA-specific probe, the Northern blot membrane was stained with 0.03% (w/v) methylene blue to confirm the integrity of the RNA and destained by 2× SSC. Following destaining, the membrane was incubated in prehybridization solution (50% formamide, 5× Denhardt's solution, 5× SSC, 25 mM Tris, pH 7.4, 0.1% SDS, and 100 μg/mL denatured herring sperm DNA) for 4 h at 42 °C with gentle shaking, followed by hybridization overnight at 42 °C with radiolabeled probe (1.2×10^9 cpm/μg) corresponding to approximately 1.0 kb of *SCR1* or *SCR2* coding sequence/mL of hybridization solution (50% formamide, 5× Denhardt's solution, 5× SSC and 25 mM Tris, pH 7.4, 0.1% SDS, 10% dextran sulfate, and 100 μg/mL denatured herring sperm DNA). Following hybridization, the membrane was washed three times for 5 min in 2× SSC and 0.1% (w/v) SDS at room temperature followed by two 20-min washes in 0.2× SSC and 0.1% (w/v) SDS at 50 °C. The membrane was subjected to autoradiography for transcript analysis.

RESULTS

Expression and Purification of Scr1 and Scr2. The *SCR1* coding sequence was subcloned into pGEX-2T and expressed in *E. coli.* The GST−Scr1 fusion protein was purified by affinity chromatography on glutathione Sepharose beads followed by thrombin digestion to remove the GST tag and then purification by Mono S FPLC (Materials and Methods). The purified Scr1 protein had an apparent molecular weight of approximately 45 kDa as judged by SDS−PAGE (Figure 1A) and was characterized as described below. A His_6 tag was attached to the N-terminus of Scr2 by subcloning *SCR2* coding sequence into pRSETA and expression in *E. coli* to produce His_6-Scr2. His_6-Scr2 was purified by a combination of Ni^+-affinity chromatography and Mono S FPLC (Materials and Methods). Purified His_6-Scr2 had an apparent molecular weight of approximately 45.5 kDa (Figure 1B).

Escherichia coli endonuclease III contains an iron−sulfur [4Fe-4S] cluster and produces a peak absorbance at 410 nm. The translated amino acid sequences of Scr1 and Scr2 predict the presence of a similar iron−sulfur cluster in Scr2 but not Scr1. Consistent with this prediction, Scr2 showed an absorbance peak at 410 nm which was not observed with Scr1 (Figure 2).

Scr1 and Scr2 Activities Are Similar to That of E. coli Endonuclease III. Escherichia coli endonuclease III acts as DNA N-glycosylase/AP lyase on a wide variety of base damage products including pyrimidine photohydrates, thymine glycol, dihydrothymine, and dihydrouracil (*5*). To determine whether Scr1 and Scr2 proteins possessed activity similar to endonuclease III, we prepared 37mer duplex oligonucleotide substrates containing each of the following modified bases: dihydrouracil (DHU-37mer), uracil (U-37mer), 8-oxoguanine (8-oxoG-37mer), and an abasic site (AP-37mer). Figure 3A shows the activities of Scr1 and Scr2 proteins on DHU-37mer. Scr1 and Scr2 mediated DNA

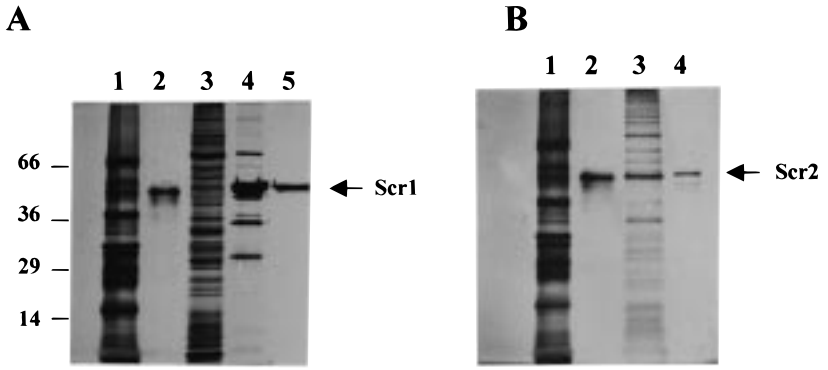

FIGURE 1: Purification of Scr1 and Scr2. Proteins were visualized on a 12% SDS−polyacrylamide gel by silver staining. (A) Scr1 expressed in *E. coli* BL21 (DE3) cells with a GST tag as described in Materials and Methods. Lane 1: protein molecular weight markers (sizes indicated on the left). Lane 2: 0.1 mg of ovalbumin (45 kDa protein standard). Lane 3: 20 μg of soluble protein from crude extract of *E. coli* expressing *SCR1*-pGEX-2T. Lane 4: 5 μg of Scr1 following removal of the GST tag (thrombin digestion) while bound to glutathione Sepharose beads. Lane 5: 1 μg Scr1 following removal of the GST tag (thrombin digestion) and further purification by Mono S FPLC (Materials and Methods). (B) Scr2 expressed in *E. coli* BL21 (DE3) cells with a polyhistidine (His_6) tag as described in Materials and Methods. Lanes 1 and 2 are the same as described in panel A. Lane 3: 10 μg of soluble protein from crude extract of *E. coli* expressing *SCR2*-pRSETA. Lane 4: 0.5 μg of Scr2 protein following elution from the Hi-Trap column and further purification by Mono S FPLC (Materials and Methods).

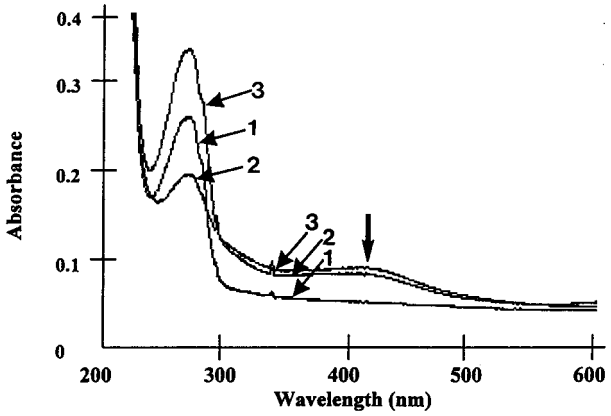

FIGURE 2: UV−visible absorption spectra of Scr1, Scr2, and *E. coli* endonuclease III. Scr1 (1), Scr2 (2), or *E. coli* endonuclease III (3) (0.5 μg each) was assayed for absorbance at 410 nm to indicate the presence or absence of the Fe−S cluster. Scr2 and endonuclease III showed an absorbance peak at 410 nm, indicated by the solid arrow. Scr1 does not absorb at this wavelength.

strand scission at the damage site with DHU-37mer in a manner that was both concentration (Figure 3A) and time (not shown) dependent. Scr1 and Scr2 cleaved AP-37mer in a manner similar to endonuclease III (Figure 3C). These results indicate that Scr1 and Scr2 possess both N-glycosylase and AP lyase activities similar to those of endonuclease III. Scr1 and Scr2 cause release of damaged bases in DNA substrates damaged with ionizing radiation as analyzed by gas chromatography/mass spectrometry, which provides additional support for the N-glycosylase activity of these proteins (unpublished). U-37mer and 8-oxoG-37mer were not cleaved by either Scr1 or Scr2, indicating that uracil and 8-oxoguanine are not substrates for these enzymes (Figure 3B,C). Single and double mutants lacking expression of Scr1 and Scr2 were constructed by insertional inactivation. These mutants were confirmed by PCR/Southern blot analysis (*24*) of the disrupted gene (not shown) and activity against known endonuclease III substrates. Extracts from either single mutant (*scr1 or scr2*) but not the double mutant showed activity against DHU-37mer (Figure 4). These results indicate that together, Scr1 and Scr2 proteins comprise the endonuclease III-like activity in *S. cerevisiae* with respect to removal of dihydrouracil from DNA.

Scr1 and Scr2 Excise FaPy-7-MeGua. Our initial studies with partially purified yeast redoxyendonuclease (*12*) and subsequently with purified Scr1 and Scr2 (unpublished) indicated these proteins were capable of recognizing and cleaving heavily UV irradiated DNA at sites of guanine. Recently, it has been shown that irradiation of DNA with high doses of UV light can result in the formation of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPy-Gua) from guanine (*25*). In addition, Eide et al. (*13*) have demonstrated that crude extracts of *E. coli* overproducing Ntg1 (Scr1) could release FaPy-7MeGua from damaged DNA but do not cleave an 8-oxoguanine-containing substrate. Therefore, we wished to determine whether homogeneous Scr1 and Scr2 could recognize and cleave DNA substrates containing FaPy-7-MeGua and the related guanine adduct 8-oxoguanine. FaPy-7-MeGua-containing plasmid DNA (pRSETA) was used in nicking assays with Scr1 and Scr2. Homogeneous Scr1, and Scr2 and formamidopyrimidine-DNA glycosylase (Fpg), but not endonuclease III, efficiently cleaved this substrate (Figure 5). Undamaged pRSETA was not cleaved by any of these proteins.

Kinetic Parameters. The V_{max} and K_m values were measured for homogeneous preparations of Scr1 and Scr2 using DHU-37mer as a substrate. Figure 6 shows the Michaelis−Menten kinetics and Lineweaver−Burk plots of the DHU-37mer cleavage reactions. The apparent K_m for DHU-37mer was calculated to be 227 ± 34 nM for Scr1 and 160 ± 20 nM for Scr2. The V_{max} values (nM min^{-1} ng^{-1}) were calculated to be 1.9 ± 0.13 for Scr1 and 0.2 ± 0.01 for Scr2. The turnover numbers (K_{cat}) were found to be 4.3 ± 0.28 min^{-1} for Scr1 and 0.5 ± 0.03 min^{-1} for Scr2. It should be emphasized that these kinetic parameters represent values obtained for enzymes purified from a bacterial overexpression system and may or may not reflect the true kinetic parameters of the native proteins expressed in yeast.

Induction of SCR1 and SCR2 by DNA Damaging Agents. The inducibility of *SCR1* and *SCR2* by reactive oxygen species (ROS) was investigated. Total RNA from yeast was

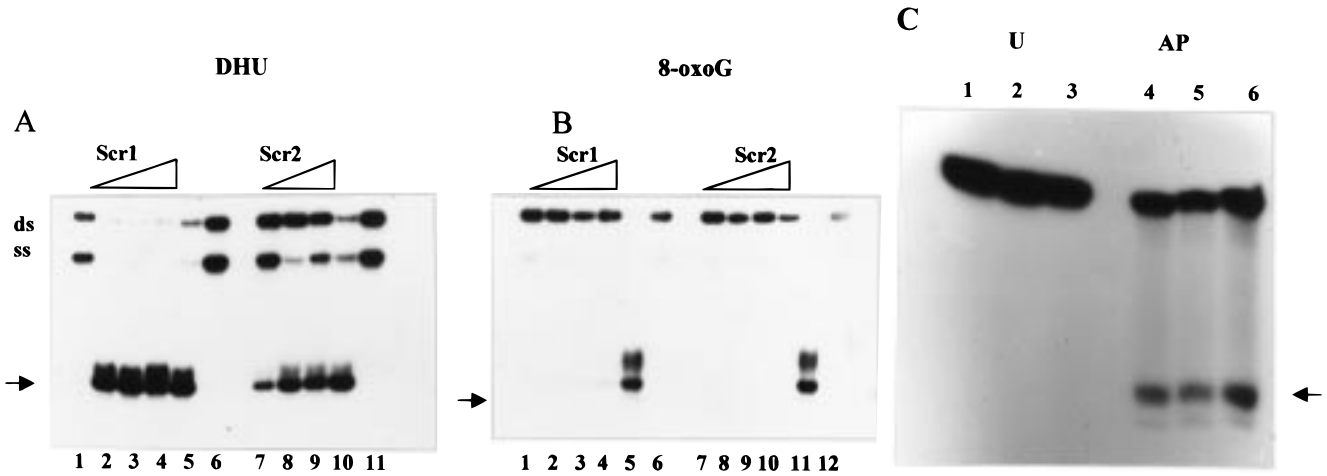

FIGURE 3: Activities of Scr1 and Scr2 on damaged DNA substrates following overexpression and purification. Oligonucleotide cleavage products were analyzed on DNA sequencing gels and subjected to autoradiography and phosphorimager analysis as described in Materials and Methods. (A) Gel autoradiogram of Scr1 and Scr2 activity monitored by incubation of 3′-end-labeled DHU-37mer with increasing amounts of Scr1 or Scr2. The DHU-37mer was incubated with 0.1, 0.5, 1, or 10 ng of Scr1 (lanes 1−4) or 0.5, 1, or 10 ng of Scr2 (lanes 7−9), respectively. The DHU-37mer was incubated with *E. coli* endonuclease III (lanes 5, 10) or buffer alone (lanes 6, 11) to serve as positive and negative controls. Double-stranded (ds) and single-stranded (ss) uncleaved DHU-37mer are indicated on the left. The arrow indicates the DNA cleavage product (16mer). (B) Gel autoradiogram of Scr1 and Scr2 activity monitored by incubation with 3′-end-labeled 8-oxoG-37mer. The 8-oxoG-37mer was incubated with 0.1, 0.5, 1, or 10 ng of Scr1 (lanes 1−4) or Scr2 (lanes 7−10), respectively. *E. coli* Fpg (lanes 5, 11) and buffer alone (lanes 6, 12) serve as positive and negative controls, respectively. The arrow indicates the DNA cleavage product (16mer). (C) Gel autoradiogram of Scr1 and Scr2 activity monitored by incubation with 3′-end-labeled U-37mer or AP-37mer. Ten nanograms of purified Scr1, Scr2, or *E. coli* endonuclease III was incubated with the U-37mer (lanes 1−3) or AP-37mer (lanes 4−6), respectively. The arrow indicates the DNA cleavage product (16mer).

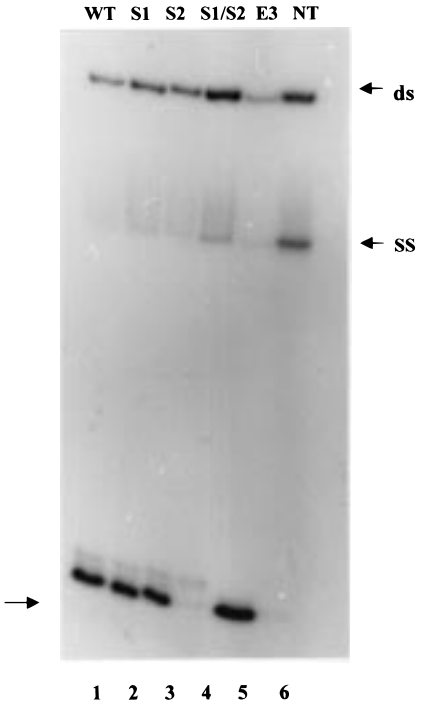

FIGURE 4: Cleavage activity on DHU-37mer by *scr* mutants. The DHU-37mer was incubated with 50 μg of total protein from crude yeast extracts (Materials and Methods). Wild-type (WT), *scr1* (S1), and *scr2* (S2) mutant strains *(SJR751, SJR832,* and *SJR835,* respectively) cleaved the DHU-37mer (lanes 1−3), indicated by the arrow. The *scr1/scr2* double mutant, *SJR834* (S1/S2), was unable to cleave the DHU-37mer (lane 4). *E. coli* endonuclease III (E3, lane 5) and buffer alone (NT, lane 6) serve as positive and negative controls, respectively. Single-stranded (ss) and double-stranded (ds) uncleaved DHU-37mer are indicated.

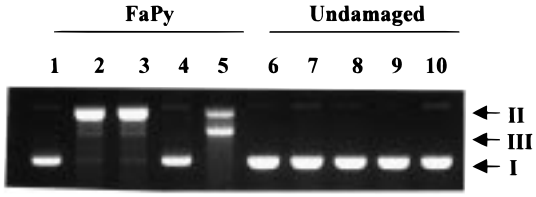

FIGURE 5: Cleavage of supercoiled pRSETA DNA containing FaPy-7-MeGua. FaPy-7-MeGua-containing (lanes 1−5) or undamaged (lanes 6−10) plasmid DNA substrates were incubated with 40 ng of Scr1 (lanes 2 and 7), Scr2 (lanes 3 and 8), endonuclease III (lanes 4 and 9), Fpg (lanes 5 and 10), or reaction buffer alone (lanes 1 and 6) and subjected to agarose gel electrophoresis as described in Materials and Methods. Arrows indicate plasmid forms I (supercoiled, covalently closed circular DNA), II (nicked, closed circular DNA), and III (linear duplex DNA).

analyzed for expression of *SCR1* and *SCR2* mRNA. The expression of *SCR1* and *SCR2* in wild-type yeast was quite low and was not detectable under our experimental conditions. However, expression of *SCR1* mRNA was detected after exposure to 0.3 mM menadione for 2 h (Figure 7). Wild-type yeast cells exposed to hydrogen peroxide did not show induction of *SCR1* (Figure 7). Under the conditions of these experiments, neither agent was found to induce *SCR2* mRNA (Figure 7). These results are in contrast to those of Eide et al. (*13*), who reported that Ntg1 (Scr1) was induced following similar exposure to hydrogen peroxide. The reason for this discrepancy is not known; however, differences in the yeast strains used between these studies might have an effect on the inducibility of these proteins as well as the exact conditions of growth used during ROS exposure.

DISCUSSION

DNA damage caused by ROS is believed to play an important role in mutagenesis and carcinogenesis (*3*, *4*). In most organisms, oxidative base damage is thought to be repaired primarily by the base excision repair (BER) pathway (*5*). BER is initiated by a DNA glycosylase, which recognizes the damaged base and cleaves its N-glycosylic

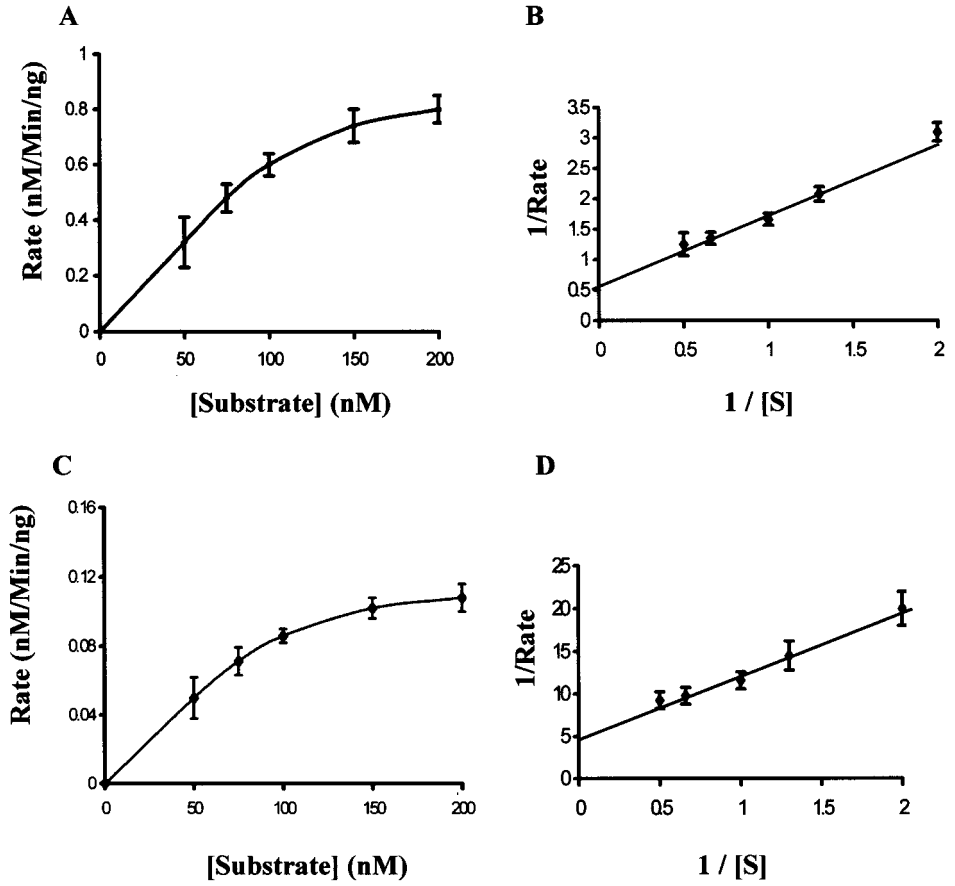

FIGURE 6: Kinetic analysis of DHU-37mer cleavage by purified Scr1 and Scr2. Scr1 (A, B) or Scr2 (C, D) was reacted with increasing amounts of 3′-end-labeled DHU-37mer and analyzed for DNA strand scission activity as described in Materials and Methods. Panels A and C are plots of the reaction rate (Rate) vs substrate concentration [S] using the mean ± standard deviation of data from four separate experiments. Curves shown are the best fit to the Michaelis−Menten equation of the averaged data. Panels B and D are the Lineweaver−Burk plots of kinetic data.

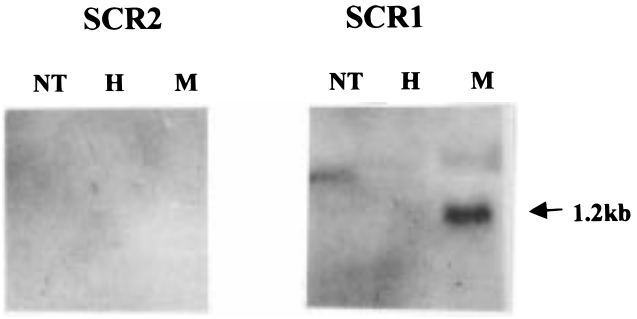

FIGURE 7: Induction of *SCR1* and *SCR2* by reactive oxygen species. Northern blot analysis was performed using 20 μg of total RNA from wild-type yeast. Cells were exposed to hydrogen peroxide (H) or menadione (M), as described in Materials and Methods, or left untreated (NT). The RNA was transferred to a nylon membrane and hybridized with the 1.0-kb ^{32}P-labeled *SCR1* or *SCR2* probe. *SCR1* mRNA (1.2 kb) was induced following exposure to menadione as indicated by the arrow. Equal amounts of RNA were loaded into each lane as determined by methylene blue staining of rRNA (not shown). The blurred horizontal region seen in the *SCR1* NT lane is an artifact and extends beyond the NT lane toward the left (not shown).

bond. *Escherichia coli* endonuclease III acts as a DNA glycosylase, removing oxidized pyrimidines from DNA, and also as an apurinic/apyrimidinic (AP) lyase which cleaves the phosphodiester backbone by β-elimination at the site where a damaged base has been removed (*6*). Endonuclease III can excise a wide range of damaged pyrimidine derivatives including thymine glycol, urea, 5,6-dihydroisobutyric acid, 5-hydroxy-6-hydrothymine, 5,6-dihydroxyuracil, and 5-hydroxy-5-methylhydantoin (*26*−*29*). Overproduction of *E. coli* endonuclease III protects against the lethal effects of ionizing radiation and chemical oxidants, and endonuclease III is considered to be an important component of the defense mechanism against oxidized pyrimidines (*30*). Genes encoding putative endonuclease III homologues have been identified in a number of species including *Bacillus subtilis* (*7*), *Haemophilus influenzae* (*8*), *Methanococcus jannaschii* (*31*), *S. pombe* (*32*), *S. cerevisiae* (*13*, *14*), and rat and human cells (*9*, *10*). Purified Scr1 and Scr2 proteins both possess N-glycosylase and AP-lyase activities against substrates containing dihydrouracil, and an *Scr1/ Scr2* double mutant had no detectable activity against the same substrate. Thus, Scr1 and Scr2 are functional homologues of endonuclease III, and *S. cerevisiae* is the first example of an organism with two such proteins. In addition, Scr1 is the only known example to date of a functional endonuclease III homologue that does not contain an iron−sulfur center. It should be pointed out that the genome of *Helicobacter pylori* contains two open reading frames which encode two putative endonuclease III homologues. However, no information exists as to whether the translated proteins actually possess endonuclease III-like activity (*33*).

Why does *S. cerevisiae* possess two homologues of *E. coli* endonuclease III? One possibility is that Scr1 and Scr2 may

act together in the nucleus for protecting against oxidative DNA base damage. Although both Scr1 and Scr2 recognize dihydrouracil, they may possess overlapping but nonidentical substrate specificities analogous to the situation with endonuclease III and endonuclease VIII in *E. coli* (*34*). Recently, two distinct DNA glycosylases that recognize 8-oxoG and are functional homologues of *E. coli* Fpg, Ogg1 and Ogg2, were identified in *S. cerevisiae* (*35*, *36*). These two enzymes have 8-oxoG glycosylase and AP lyase activities. However, the substrate specificities of these two proteins are somewhat different; Ogg1 removes the 8-oxoG paired opposite pyrimidines, but Ogg2 removes the 8-oxoG from an 8-oxoG/A mispair (*37*). Similarly, Scr1 and Scr2 may possess substrate preferences for damaged pyrimidines within different base pair contexts, although there is currently no evidence to support this notion. An additional possibility is that Scr1 and Scr2 may have different subcellular locations. Endonuclease III homologues possess two conserved sequences, an iron−sulfur cluster and a helix−hairpin−helix motif, that have been shown to have a DNA binding role (*11*). Interestingly, the iron−sulfur cluster is not found in Scr1. However, the N-terminus of Scr1 encodes a hydrophilic region that could function as a mitochondrial transit peptide (*38*). Another enzyme, uracil-DNA glycosylase (UNG), has been shown to exist in a nuclear and mitochondrial form in mammalian cells (*39*). The nuclear (UNG2) and mitochondrial (UNG1) forms differ in their N-terminal amino acid sequences that direct nuclear and mitochondrial importation (*40*). A similar situation may exist for the Scr1 and Scr2 proteins if Scr1 is directed to the mitochondria and Scr2 is imported to the nucleus. However, it is also conceivable that both proteins exist in both the nucleus and mitochondria.

Northern blot analysis indicates that *SCR1* and *SCR2* mRNAs are present at very low (undetectable) levels under normal growth conditions. However, both mRNAs can be detected using RT−PCR methodologies (unpublished). The level of *SCR1*, but not *SCR2*, mRNA is elevated following menadione exposure. We currently do not know the relationship between mRNA and protein levels for these enzymes, although activities corresponding to both Scr1 and Scr2 are easily detected under normal growth conditions (Figure 4).

In eukaryotes, oxidative phosphorylation occurs in the mitochondria to produce ATP. During this process, ROS can be produced with the potential for damaging mitochondrial DNA (*41*, *42*). However, mitochondrial DNA is more vulnerable to attack by ROS due to its lack of histones. The repair capacity of mitochondria for oxidative damage appears to be less than that of the nucleus, and mitochondrial DNA damage is more extensive and persistent than nuclear DNA damage (*43*). Thus, mitochondrial DNA is a critical target for ROS. Oxidative DNA damage in the mitochondria has been linked to the onset of specific human diseases such as neuronal degeneration, cardiovascular disease, and aging (*44*−*46*). We speculate that the inducibility of Scr1 may have a role in protecting mitochondrial DNA against ROS. Until now, only iron−sulfur-containing endonuclease III homologues have been found in eukaryotes, with the exception of Scr1 in *S. cerevisiae*. Thus, some organisms may possess multiple endonuclease III homologues that are specific for the repair of nuclear and mitochondrial DNA and which may have overlapping but nonidentical substrate specificities.

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