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Saccharomyces cerevisiae Ntg1p and Ntg2p: Broad Specificity *N*-Glycosylases for the Repair of Oxidative DNA Damage in the Nucleus and Mitochondria†

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ABSTRACT: *Saccharomyces cerevisiae* possesses two functional homologues (Ntg1p and Ntg2p) of the *Escherichia coli* endonuclease III protein, a DNA base excision repair *N*-glycosylase with a broad substrate specificity directed primarily against oxidatively damaged pyrimidines. The substrate specificites of Ntg1p and Ntg2p are similar but not identical, and differences in their amino acid sequences as well as inducibility by DNA damaging agents suggest that the two proteins may have different biological roles and subcellular locations. Experiments performed on oligonucleotides containing a variety of oxidative base damages indicated that dihydrothymine, urea, and uracil glycol are substrates for Ntg1p and Ntg2p, although dihydrothymine was a poor substrate for Ntg2p. Vectors encoding Ntg1p-green fluorescent protein (GFP) and Ntg2p-GFP fusions under the control of their respective endogenous promoters were utilized to observe the subcellular targeting of Ntg1p and Ntg2p in *S. cerevisiae*. Fluorescence microscopy of pNTG1-GFP and pNTG2-GFP transformants revealed that Ntg1p localizes primarily to the mitochondria with some nuclear localization, whereas Ntg2p localizes exclusively to the nucleus. In addition, the subcellular location of Ntg1p and Ntg2p confers differential sensitivities to the alkylating agent MMS. These results expand the known substrate specifities of Ntg1p and Ntg2p, indicating that their base damage recognition ranges show distinct differences and that these proteins mediate different roles in the repair of DNA base damage in the nucleus and mitochondria of yeast.

Reactive oxygen species (ROS) produced in cells by aerobic metabolism or due to exposure to ionizing radiation or chemical oxidants cause a wide variety of DNA base damage (*1*). Such damage, if left unrepaired, has been implicated in a number of degenerative processes including cancer and aging (*2*−*4*). In the majority of species, the repair of oxidative DNA base damage is thought to be primarily mediated by the base excision repair (BER) pathway and, depending on the nature of the lesion, is initiated by various DNA *N*-glycosylases. In *Escherichia coli*, three *N*-glycosylases, endonuclease III (endo III),[1] endonuclease VIII (endo VIII), and formamidopyrimidine glycosylase (Fpg), are responsible for recognizing and removing the majority of oxidatively damaged purines (Fpg) and pyrimidines (endo III and endo VIII) via combined *N*-glycosylase/AP lyase activities (*1*). Functional homologues of these proteins have been found in a large number of organisms including yeast and man (*5*−*8*).

Saccharomyces cerevisiae possesses two functional homologues of endo III encoded by the *NTG1* and *NTG2* genes, which are nonessential for growth. The encoded proteins Ntg1p and Ntg2p are comprised of 399 and 380 amino acids, respectively, and show significant sequence similarity to endo III (24% identity, 46% similarity to Ntg1p; 25% identity, 51% similarity to Ntg2) as well as to each other (41% identity, 63% similarity) (*7*). Endo III and its homologues in other species possess two highly conserved regions: a putative active site helix−hairpin−helix (HhH) domain and an iron−sulfur cluster located near the C-terminus (*5*−*8*).

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[1] Abbreviations: 5-OH-Cyt, 5-hydroxycytosine; 5-OH-Ura, 5-hydroxyuracil; 8-OH-Gua, 8-hydroxyguanine; DHT, dihydrothymine; DHU, dihydrouracil; endo III, endonuclease III; endo VIII, endonuclease VIII; Ntg, endonuclease III-like glycosylase; GC/IDMS, gas chromatography/isotope dilution mass spectrometry; Thy-gly, thymine glycol; Ura-gly, uracil glycol.

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Table 1: Oligonucleotides Used in This Study[a]

oligo	sequence
DHU 37mer[b]	5′-CTTGGACTGGATGTCGGCAC[**U**[a]]AGCGGATACAGGAGCA-3′
8-OH-Gua 37mer	5′-CTTGGACTGGATGTCGGCAC[**G**[a]]AGCGGATACAGGAGCA-3′
Ura-gly 45mer[b]	5′-GCAGCCAAAACGTCC[**U**[b]]GGATGGTCTGTCCCTTGAATCGATAGGGG-3′
5-OH-Cyt 54mer[b]	5′ ATTCCAGACTGTCAATAACACGG[**C**[a]]GGACCAGTCGATCCTGGGCTGCAGGAATTC-3′
5-OH-Ura-54mer[b]	5′ ATTCCAGACTGTCAATAACACGG[**U**[c]]GGACCAGTCGATCCTGGGCTGCAGGAATTC-3′
Thy-gly 54mer[c]	5′ ATTCCAGACTGTCAATAACACGG[**T**[a]]GGACCAGTCGATCCTGGGCTGCAGGAATTC-3′
DHT 54mer[c]	5′ ATTCCAGACTGTCAATAACACGG[**T**[b]]GGACCAGTCGATCCTGGGCTGCAGGAATTC-3′
urea 54mer[c]	5′ ATTCCAGACTGTCAATAACACGG[**Ur**]GGACCAGTCGATCCTGGGCTGCAGGAATTC-3′

[a] Oligonucleotides containing various DNA base damages were synthesized as described in Experimental Procedures. Base U[a], G[a], U[b], and U[c] denote DHU, 8-OH-Gua, Ura-gly, and 5-OH-Ura, respectively. C[a] denotes 5-OH-Cyt. T[a] and T[b] denote Thy-gly and DHT, respectively. Ur denotes urea. Complementary strands are not shown. [b] The complementary strand contains G opposite to the lesion. [c] The complementary strand contains A opposite to the lesion.

Although both Ntg1p and Ntg2p contain the HhH domain, only Ntg2p possesses the iron−sulfur center (*7, 9*). Furthermore, Ntg1p possesses a long positively charged N-terminus which has been identified as a putative mitochondrial transit sequence, raising the possibility that Ntg1p is involved in the repair of mitochondrial oxidative DNA damage (*7, 9*). Recent studies of Ntg1p and Ntg2p have revealed that both proteins recognize and excise a similar spectrum of base damage products including thymine glycol, dihydrouracil, 5-hydroxy-6-hydrothymine (5-OH-6-HThy), 5-hydroxy-6-hydrouracil (5-OH-6-HUra), 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd), 5-hydroxyuracil (5-OH-Ura), 5-hydroxycytosine (5-OH-Cyt), 2,6-diamino-4-hydroxy-5-(*N*-methylformamido)pyrimidine (Fapy-7MeGua), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 4,6-diamino-5-formamidopyrimidine (FapyAde), and abasic sites (*9, 10*). One group has reported that Ntg1p also recognizes and excises 8-hydroxyguanine (8-OH-Gua) paired with any of the four DNA bases and that this protein corresponds to yOgg2p which has been proposed to mediate a major role in the yeast "GO" system for reversing the potential mutagencity of 8-OH-Gua in DNA (*11*). This result is in contrast to several studies showing lack of Ntg1p-mediated excision of 8-OH-Gua paired with cytosine, adenine, or thymine in either oligonucleotides or irradiated calf thymus DNA, although Ntg1p does remove 8-OH-Gua at a low efficiency when paired opposite to guanine (*5, 10*).

To gain insight into the potential biological roles of Ntg1p and Ntg2p, we have conducted a series of experiments focused on revealing differences between these two DNA repair enzymes. To examine the subcellular distribution of these two proteins, Ntg1p-green fluorescent protein (GFP) and Ntg2p-GFP fusions were expressed in wild-type yeast, and the subcellular localization of Ntg1p and Ntg2p was determined by fluorescence microscopy. Recent studies have shown that the human homologue of endo III, hNth1, is apparently localized to both the mitochondria and the nucleus when transiently expressed in COS-7 or HeLa cells (*12*). Interestingly, hNth1 is more similar to Ntg2p in *S. cerevisiae* which we demonstrate is only localized to the nucleus.

In addition to examining the localization of the Ntg proteins, we have addressed the role that this subcellular localization of Ntg1p and Ntg2p plays in cellular sensitivity to DNA damaging agents. In vitro studies with homogeneous Ntg1p and Ntg2p and oligonucleotides containing various oxidative base damages were conducted to indicate potential differences in their substrate specificities. Our results show that Ntg1p and Ntg2p are targeted to different subcellular locations and exhibit several important differences with respect to their substrate specificities.

EXPERIMENTAL PROCEDURES

Materials. Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Polyclonal antibodies against GFP and Npl3p were generous gifts from Drs. J. Kahana and P. Silver (*13, 14*). *E. coli* endonuclease III and endonuclease VIII were prepared and purified as previously described (*15, 16*). *E. coli* Fpg protein was a gift from Dr. Yoke Wah Kow (Atlanta, GA). *S. cerevisiae* Ntg1p and Ntg2p were generated and purified as previously described (*9, 10*).

Strains and Vectors. The pPS904 GFP expression vector (*17*) was employed for generation of Ntg1p-GFP and Ntg2p-GFP fusions. *S. cerevisiae* haploid strain ACY193 (FY86) (*MAT*a *ura3-52 leu2*Δ*1 his3*Δ*200*) was utilized for all localization studies (*18*). Isogenic derivatives of wild-type strain SJR751 (*MAT*α *ade2-101 his3*Δ*200 leu2-R ura3*Δ*Nco lys2*Δ*Bgl*) were used for MMS sensitivity studies: SJR832 (*ntg1* mutant), SJR835 (*ntg2* mutant), and SJR834 (*ntg1 ntg2* mutant) were constructed as previously described (*9*). SJR751, SJR832, SJR835, and SJR834 were transformed by the lithium acetate protocol (*19*) with *Eco*RI−*Bam*HI-digested pSCP19A (*20*) to introduce *apn1*Δ*::HIS3*. His$^+$ transformants were selected on synthetic complete medium (*21*) containing 2% dextrose and lacking histidine (SD-HIS) to yield *apn1* (SJR864), *ntg1 apn1* (SJR865), *ntg2 apn1* (SJR866), and *ntg1 ntg2 apn1* (SJR867) mutants. All yeast strains were maintained on YEPD medium (1% yeast extract, 2% Bacto-peptone, 2% dextrose; 2.5% agar for plates) unless otherwise noted.

Oligonucleotides. The sequences of the oligonucleotides used in this study are shown in Table 1. Oligonucleotides DHU 37mer and 8-OH-Gua 37mer were synthesized, end-labeled, and annealed to the proper complementary strands, and the respective duplex oligonucleotides were purified on nondenaturing polyacrylamide gels as previously described (*7, 9*). Oligonucleotides containing uracil glycol (Ura-gly 45mer), 5,6-dihydrothymine (DHT 54mer), urea (urea 54mer), thymine glycol (Thy-gly 54mer), 5-OH-Cyt (5-OH-Cyt 54mer), and 5-OH-Ura (5-OH-Ura 54mer) and the appropri-

ate corresponding complementary strands (Table 1) were synthesized as previously described (*22*−*26*). Oligonucleotides were either 5′-end-labeled using [γ-^{32}P]ATP (ICN, 3000 Ci/mmol) and T4 polynucleotide kinase (New England Biolabs) and annealed with the appropriate complementary strand as previously described (*26*) or 3′-end-labeled with [α-^{32}P]dideoxyATP (Amersham, 300 Ci/mmol) and terminal deoxynucleotidyl transferase (Promega) and annealed to the appropriate complementary strand as previously described (*9*).

Generation of Ntg-GFP Fusion Proteins. A 2.2-kb insert containing the endogenous promotor and the entire *NTG1* structural gene was generated by PCR from genomic yeast DNA using the primers 5′-AACTGCAGGCTGAGACTTTACAATGG-3′ and 5′-CCGCTCGAGCGTCCTCTACTTTAACAGAAATATC-3′, and a 2.15-kb insert containing the endogenous promotor and the entire *NTG2* structural gene was generated by PCR from genomic yeast DNA using the primers 5′-AACTGCAGGGTAGGAGCTGCTTAATG-3′ and 5′-CCGCTCGAGCTTTTTTCTTGTCTTTCTGTTG-3′. The PCR products were digested with *Pst*I and *Xho*I and were subcloned into the *Pst*I and *Xho*I sites of the GFP-containing vector pPS904 (*17*). The resulting plasmids, pNTG1-GFP and pNTG2-GFP, contained *NTG1* and *NTG2* fused in-frame at their respective C-termini (at the *Xho*I site) to a GFP gene leader sequence, respectively. pNTG1-GFP and pNTG2-GFP were transformed into *S. cerevisiae* strain ACY193 using a lithium acetate method (*19*). Transformants were selected on synthetic complete medium (*21*) lacking uracil and containing 2% dextrose (SD-URA). Expression of functional GFP fusion proteins was confirmed by Western blot analysis and Ntg protein activity assays as described below.

Subcellular Localization of Ntgp-GFP Fusions by Fluorescence Microscopy. The subcellular locations of Ntg1p and Ntg2p were determined by fluorescence microscopic analysis of pNTG1-GFP and pNTG2-GFP transformants. Yeast cells were grown in SD-URA medium at 30 °C to a concentration of 1×10^7 cells/mL. For visualization of mitochondria, MitoTracker Red CMXRos (Molecular Probes) was added to the culture media and cells were grown for an additional 30 min. Aliquots (10 μL) of living cells were examined for fluorescence through a GFP optimized filter (Chroma Technology) or for the MitoTracker signal through a Texas Red/rhodamine filter on an Olympus BX60 microscope (Melville, NY) equipped with a Photometrics Quantix (Tuscon, AZ) digital camera. Indirect immunofluorescence microscopy was employed to confirm the nuclear localization of Ntg2p. For these experiments cells were grown in SD-URA at 30 °C to a concentration of 1×10^7 cells/mL and were prepared for indirect immunofluorescence microscopy as described previously (*13*). Briefly, cells were fixed in 3.7% formaldehyde for 30 min, washed in 0.1 M potassium phosphate buffer, pH 6.5, and resuspended in P solution (0.1 M potassium phosphate buffer, pH 6.5, 1.2 M sorbitol). Cells were then spheroplasted with 15−30 mg of Zymolyase (10 mg/mL in P solution) and with 25 μL of 1 M DTT. Digestion was monitored by observing the cells under phase microscopy. Following digestion, cells were adhered to slides pretreated with 0.3% polylysine. After adherence, cells were fixed in methanol for 6 min at −20 °C, and then slides were dried in cold acetone for 30 s followed by blocking the slides once with PBS−BSA (5 mg/mL BSA in phosphate-buffered saline) before incubation with antibody. Incubation with antibodies against Npl3p (1:500 dilution) was followed by incubation with FITC-labeled anti-rabbit antibodies (Jackson ImmunoResearch) at a 1:1000 dilution and with 4,6-diamido-2-phenylindole (DAPI) (1 mg/mL).

Immunoblots. Cells were grown under conditions used for fluorescence microscopy studies. Cell pellets were washed once in water and resuspended in 2.5× standard protein sample buffer (28) containing protease inhibitors (0.5 mM PMSF and 3 μg/mL each leupeptin, aprotinin, and pepstatin). Cells were lysed by glass bead smash and centrifuged, and the supernatant was heated at 95 °C for 5 min and subjected to SDS−PAGE (10% gels). Transfer of protein and immunoblotting were carried out as previously described (*29*). To detect the presence of Ntg1p-GFP, Ntg2p-GFP or GFP alone, blots were probed with a polyclonal anti-GFP antibody (1:5000 dilution). Immunoreactive bands were visualized with an ECL chemiluminescence kit (Amersham Life Sciences, Inc.).

Expression and Activity of Ntg-GFP Fusions in Wild Type and ntg Mutant Strains. The Ntg1-GFP or Ntg2-GFP containing pPS904 vector was transformed into an *ntg1*, *ntg2*, or *ntg1 ntg2* mutant strain using a lithium acetate protocol (*19*). Transformants were selected on SD-URA medium. Samples were grown as described for fluorescence microscopy. Yeast cells were pelleted and then resuspended in 50 mM KH_2PO_4 (pH 7.0) containing protease inhibitors (0.5 mM PMSF and 3 μg/mL each leupeptin, aprotinin, and pepstatin). The yeast cell suspension was disrupted by vortexing (five 30-s rounds) with an equal volume of glass beads in microcentrifuge tubes and held on ice for 60 s between bursts, and then cell debris was removed by centrifugation. The crude extract (50 μg) was incubated with a 3′-end-labeled dihydrouracil (DHU)-containing oligomer (DHU 37mer) duplex oligonucleotide in a 50 μL reaction mixture containing buffer B (15 mM KH_2PO_4, pH 6.8, 10 mM EDTA, 10 mM β-mercaptoethanol) plus 40 mM KCl for 30 min at 37 °C. DNA was extracted with PCIA and ethanol-precipitated, and the reaction products were analyzed on DNA sequencing gels as previously described (*7*). 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).

Methylmethanesulfonate Survival Assays. Methylmethanesulfonate (MMS) survival assays were performed as described previously (*30*). Briefly, cells grown overnight in 5 mL of YEPD were pelleted, washed twice in sterile distilled H_2O, and resuspended in 5 mL of 1× PBS. Aliquots of cells were then subjected to the indicated concentrations of MMS for 1 h at 30 °C with shaking. The cells were then diluted in sterile distilled H_2O and plated onto YEPD medium, and colonies were counted after 3 days at 30 °C.

GC/IDMS. Oligo 8-OH-Gua 37mer was annealed to four different complementary strands containing C, G, A, or T paired opposite to 8-OH-Gua. Each of the resulting four different 8-OH-Gua-containing duplexes (5 nmol) was incubated (in triplicate separate reactions) with 1 μg of Ntg1p in 50 mM KH_2PO_4 (pH 7.5), 1 mM EDTA, and 100 mM KCl (100 μL final volume) at 37 °C for 30 min. Similar

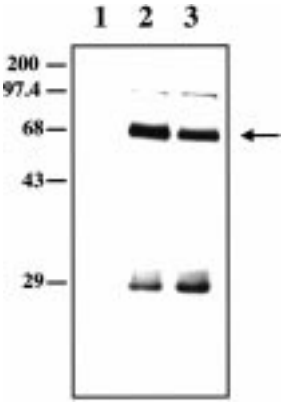

FIGURE 1: Expression of Ntg1p-GFP and Ntg2p-GFP fusion proteins. Cell extracts from the yeast strain ACY193 expressing Ntg1-GFP (lane 2) or Ntg2-GFP (lane 3) or those lacking an Ntg-GFP construct (lane 1) were analyzed on a 10% SDS−polyacrylamide gel. Following protein transfer, the blots were probed with a polyclonal antibody to GFP and subjected to ECL chemiluminescence as described in Experimental Procedures. The Ntg-GFP fusion proteins are indicated by the arrow. The cross-reacting band at ~29 kDa is GFP. Molecular weight markers are indicated on the left.

experiments were carried out using *E. coli* Fpg protein instead of Ntg1p. Following heat inactivation of Ntg1p or Fpg protein, the oligomers were ethanol precipitated. The supernatants and pellets were separated.

Aliquots of the stable isotope-diluted analogue of 8-OH-Gua were added as internal standards to pellets with known DNA amounts and to supernatant fractions. Pellets were dried in vacuo in a SpeedVac and then hydrolyzed with 0.5 mL of 60% formic acid in evacuated and sealed tubes at 140 °C for 30 min. The hydrolysates were lyophilized in vials for 18 h. Supernatant fractions were freed of ethanol in vacuo and subsequently lyophilized for 18 h. Supernatant fractions and hydrolyzed pellets were derivatized and analyzed by GC/IDMS as previously described (*31*, *32*).

RESULTS

Expression of Ntg-GFP Fusion Proteins. Expression of Ntg1p-GFP and Ntg2p-GFP fusion proteins was confirmed by Western blotting as described in Experimental Procedures. Briefly, cells were lysed in Laemmli buffer by glass bead smash, heated at 95 °C for 5 min, and resolved by SDS−PAGE (10% gel). Following transfer by standard methods (*29*), the blots were probed with a polyclonal anti-GFP antibody and visualized using ECL chemiluminescence. As observed in Figure 1, cells expressing either Ntg1p-GFP (lane 2) or Ntg2-GFP (lane 3) show fusion protein production while the untransformed yeast cells (lane 1) do not.

Ntg1p-GFP and Ntg2p-GFP Localization by Fluorescence Microscopy. Vector constructs containing Ntg1p or Ntg2p fused with GFP were transformed into the yeast strain ACY193 as described in Experimental Procedures. As shown in Figure 2A, Ntg1p localizes to both the nucleus and the mitochondria (panels A and E), while Ntg2p is only observed in the nucleus (panels I and M). Mitochondrial localization was confirmed by the use of MitoTracker Red (panels B, F, J, and N), and the merged images of GFP and MitoTracker demonstrate Ntg1p localization to both the nucleus and the mitochondria (panels C and G), while Ntg2p is only in the nucleus (panels K and O). As seen in panels E, F, and G, Ntg1p is found exclusively in the mitochondria of certain cells. This exclusive localization of Ntg1p to the mitochondria was observed in approximately 43−47% of yeast cells in our nonsynchronous population, indicating possible cell cycle regulation of Ntg1p localization.

To confirm that Ntg2p is a strictly nuclear protein, immunofluorescence using an antibody to a nuclear protein, Npl3p, was utilized (*13*). As seen in Figure 2B, Ntg2p-GFP localizes to the nucleus (panels A and F). DAPI staining of nuclear DNA is observed in panels B and G, while the nuclear protein Npl3 can be observed in panels C and H. A merged image of all three markers (panels D and I) shows that Ntg2p is only found in the nucleus as no other signal outside of the nucleus is observed. We conclude that Ntg1p is targeted to both the nucleus and the mitochondria, and Ntg2p is only targeted to the nucleus.

Activity of the Ntg-GFP Fusion Proteins. To confirm that the Ntg proteins fused to GFP are active in vivo, the Ntg1p-GFP or the Ntg2p-GFP construct was transformed into *ntg1*, *ntg2*, or *ntg1 ntg2* yeast mutants. Cell extracts were incubated with 3′-end-labeled DHU 37mer in the presence of 10 mM EDTA to prevent nonspecific exonuclease degradation. The DNA was extracted, ethanol precipitated, and run on denaturing (7 M urea) polyacrylamide DNA sequencing gels as described in Experimental Procedures. Previously, we have shown that cell extracts from either the *ntg1* or *ntg2* single mutant are able to cleave a DHU-containing oligonucleotide substrate (Table 1), while the *ntg1 ntg2* double mutant does not possess any ability to act on this substrate (*9*). This result is confirmed in Figure 3, lanes 1−3, respectively. However, when the *ntg1 ntg2* mutant is transformed with either the Ntg1p-GFP or the Ntg2p-GFP construct, activity against the DHU-containing substrate is restored (Figure 3, lanes 6 and 9, respectively). This demonstrates that the Ntg-GFP fusion proteins are active and that the targeting of the fusion proteins observed by fluorescence microscopy is representative of the localization of the native proteins in vivo.

Sensitivity of the Ntg Mutants to DNA Damaging Agents. To examine any potentially deleterious biological effects due the loss of the Ntg1 and Ntg2 proteins, yeast disruption mutants lacking one or both of these proteins were constructed as previously described (*9*). The *ntg1* mutant does not show an increased rate of petite formation (*33*), nor does it demonstrate an increased rate of mitochondrial DNA mutation as measured by erythromycin resistance (*34*, *35*) (data not shown). Interestingly, none of the mutants (*ntg1* and *ntg2* single mutants or the *ntg1 ntg2* double mutant) exhibited any increased sensitivity to DNA damaging agents which produce oxidative base damage products recognized in vitro by the Ntg proteins (H_2O_2, ionizing radiation, or UV irradiation) (data not shown). On the basis of the known *N*-glycosylase/AP lyase activity mediated by Ntg1p and Ntg2p (*9*), we postulated that the mutants might be sensitive to MMS, an alkylating agent that produces a high level of abasic sites (*36*). However, the *ntg1* single mutant, *ntg2* single mutant, and the *ntg1 ntg2* double mutant were not sensitive to MMS compared to the wild-type strain (Figure 4 and data not shown). We then disrupted the major AP endonuclease (*APN1*) in combination with the Ntg proteins in order to further decrease the ability of these cells to repair AP sites. The *ntg1 ntg2 apn1* triple mutant was found to be no more sensitive to oxidizing agents (H_2O_2, menadione, or

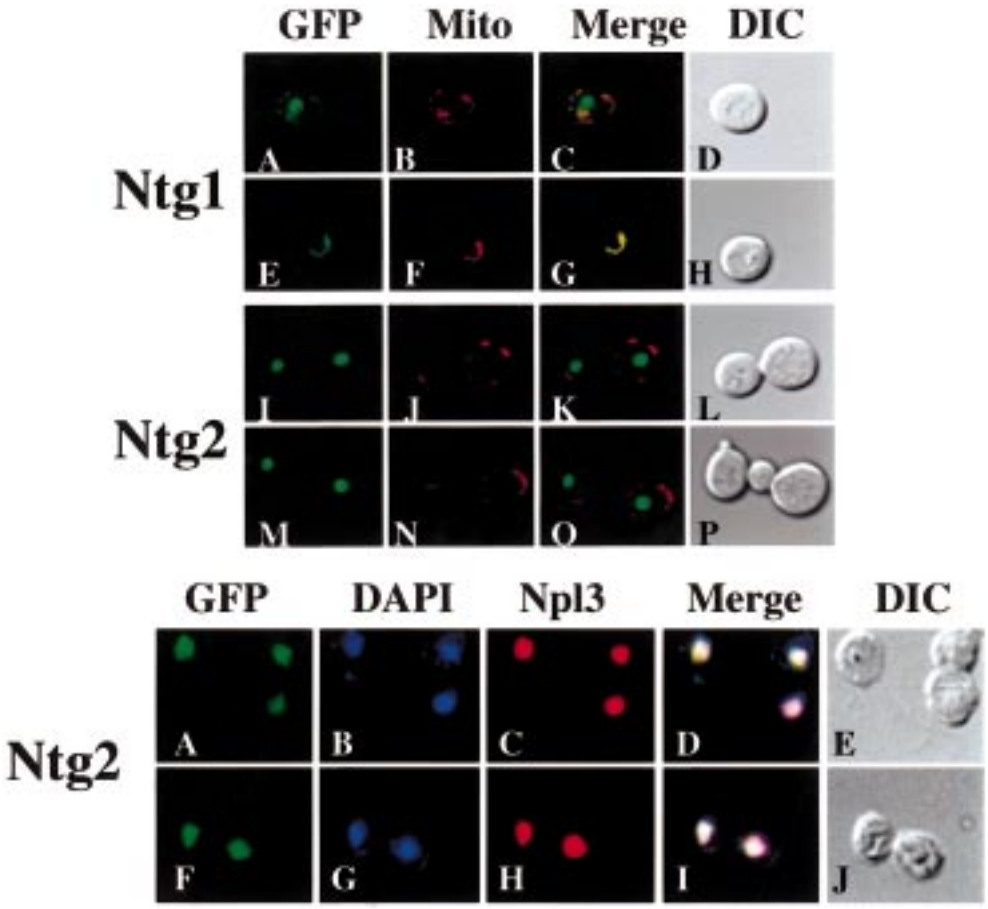

FIGURE 2: Subcellular localization of Ntg-GFP fusion proteins. (A, top) ACY193 cells expressing Ntg1p-GFP (panels A−H) or Ntg2p-GFP (panels I−P) were grown as described in Experimental Procedures. The localization of Ntg1p-GFP (panels A and E) or Ntg2p-GFP (panels I and M) was visualized using a GFP optimized filter. To visualize mitochondria, MitoTracker Red was viewed through a Texas Red/rhodamine filter (panels B, F, J, and N). The merged yellow images for Ntg1p-GFP (panels C and G) show localization to both the mitochondria and the nucleus, while the merged images for Ntg2p-GFP (panels K and O) show localization only to the nucleus. Panels D, H, L, and P show differential image contrast (DIC) images of the visualized yeast cells. (B, bottom) ACY193 cells expressing Ntg2p-GFP were fixed in methanol prior to incubation with DAPI and the Npl3p antibody as described in Experimental Procedures. As seen in panels A and F, Ntg2p-GFP was localized in the nucleus, confirmed by DAPI staining of the nuclear DNA (panels B and G). The nuclear protein Npl3 was visualized using a FITC-labeled antibody (panels C and H). The merged images (pink) of GFP, DAPI, and Npl3p (panels D and I) demonstrate localization of Ntg2p-GFP to the nucleus. Panels E and J show DIC images of the visualized yeast cells.

ionizing radiation) than wild-type cells, and in order to observe sensitivity to oxidizing agents in *S. cerevisiae*, multiple DNA repair pathways must be knocked out (*37*). However, when we examined the sensitivity of the mutants lacking the Ntg proteins and Apn1p, we found the mutant cells were more sensitive to MMS than wild-type cells (Figure 4). The loss of Apn1p alone only had a small effect on cell survival; however, the *ntg1 ntg2 apn1* triple mutant displayed a significantly increased sensitivity to MMS (Figure 4). Furthermore, although the *ntg1 apn1* double mutant displayed an MMS sensitivity similar to that of the *apn1* single mutant, the *ntg2 apn1* double mutant was more sensitive to the killing effects of MMS. These results suggest that Ntg2p is more efficient at initiating the repair of AP sites than Ntg1p, or alternatively, the differential subcellular localization of each of the two proteins plays a role in the sensitivity to MMS.

Substrate Specificity. Previously, we have shown that recombinant Ntg1p and Ntg2p excise similar DNA base lesions from γ-irradiated DNA with different efficiencies (*10*). Both enzymes will remove a variety of damaged pyrimidines; however, Ntg1p is able to remove 5-hydroxy-5-methylhydantoin as well as 8-OH-Gua (when paired opposite to guanine), while Ntg2p cannot remove either of these damages (*10*). In addition to further examining the substrate specificity of Ntg1p and Ntg2p, we wished to compare the enzyme activities to those of their well-characterized bacterial homologues , endo III and endo VIII. We utilized a variety of damage-containing oligonucleotides (Table 1) to further investigate the substrate specificities of the Ntg proteins, some of which are known substrates for the Ntg proteins (5-OH-Cyt, 5-OH-Ura, and Thy-gly) and others which are novel substrates for Ntg1p and Ntg2p (DHT, urea, and Ura-gly). To compare the respective abilities of these proteins to recognize a particular damage with their bacterial functional homologues, we also treated the damaged oligonucleotides with *E. coli* endo III or endo VIII. As seen in Figure 5, Ntg1p and Ntg2p have similar substrate specificities, but there is at least one notable difference. Dihydrothymidine (DHT) is recognized by Ntg1p and endo VIII but poorly recognized by endo III and Ntg2p. 5-hydroxycytosine (5-OH-Cyt) was recognized and removed by all four proteins as was 5-hydroxyuracil (5-OH-Ura), urea, thymine glycol (Thy-gly), and uracil glycol (Ura-gly). These results indicate qualitative similarities and differences among Ntg1p, Ntg2p, endo III, and endo VIII with these substrates.

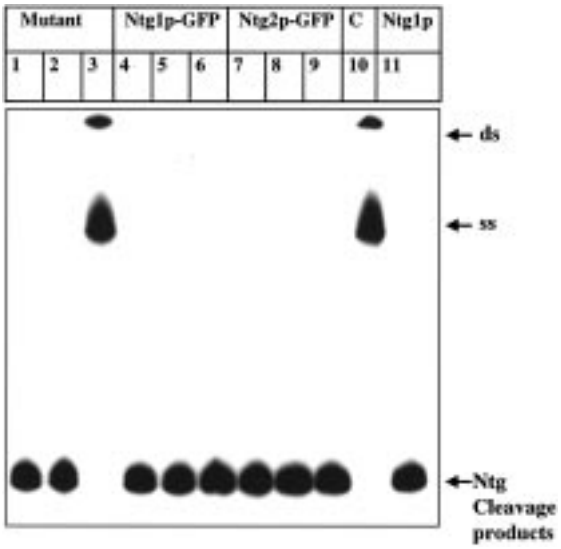

FIGURE 3: Activity of Ntg-GFP fusion proteins against a DHU-containing oligonucleotide. Cell extracts from yeast *ntg1*, *ntg2*, and *ntg1 ntg2* mutants (lanes 1−3) and those same mutants expressing Ntg1p-GFP (lanes 4−6) or Ntg2p-GFP (lanes 7−9) were incubated with duplex 3′-end-labeled DHU 37mer and run on a denaturing (7 M urea) polyacrylamide DNA sequencing gel as described in Experimental Procedures. Cell extracts from *ntg1* and *ntg2* single mutants show cleavage at DHU generating a 16 nt DNA strand scission product (indicated by the lower arrow, lanes 1 and 2, respectively), while the *ntg1 ntg2* double mutant no longer cleaves this substrate (lane 3). When Ntg1p-GFP is expressed in the *ntg1*, *ntg2*, or *ntg1 ntg2* mutant (lanes 4−6, respectively), cleavage of the DHU substrate is restored. Similarly, when Ntg2p-GFP is expressed in the *ntg1*, *ntg2*, or *ntg1 ntg2* mutant (lanes 7−9, respectively), activity against the DHU substrate is restored. Substrate incubated with buffer only or purified Ntg1p is seen in lanes 10 and 11, respectively.

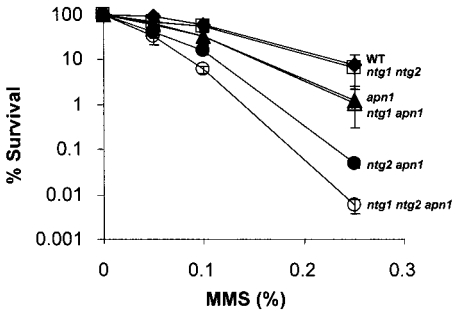

FIGURE 4: Sensitivity of *ntg* mutant strains to MMS. Cells were exposed to increasing concentrations of MMS (0−0.3%) for 60 min. The number of surviving colonies was monitored following 2−4 days of growth at 30 °C as described in Experimental Procedures. Curves: WT (◆), *ntg1 ntg2* (□), *apn1* (▲), *ntg1 apn1* (△), *ntg2 apn1* (●), and *ntg1 ntg2 apn1* (○). Error bars represent the standard deviation between three independent experiments.

Figure 5 also demonstrates that Ntg1p, Ntg2p, and endo III generate the same reaction product via β-elimination reaction, whereas endo VIII incises DNA via β- and δ-elimination reactions. We conclude that Ntg1p and Ntg2p recognize similar base lesions, but recombinant Ntg1p appears to be more efficient at the removal of particular damages compared to recombinant Ntg2p.

Several recent studies have reported conflicting results regarding the ability of Ntg1p to excise 8-OH-Gua from DNA (*5*, *9*−*11*). These studies employed various techniques such as sequencing gel analysis of DNA strand scission products, borohydride trapping, and HPLC with electrochemical detection. Using a different technique, GC/IDMS, we also investigated the removal of 8-OH-Gua by Ntg1p from duplex oligonucleotides containing 8-OH-Gua paired opposite to each of the four DNA bases (A, T, G, and C). No sigificant excision of 8-OH-Gua by Ntg1p on these substrates was observed, whereas substantial excision of 8-OH-Gua was observed by *E. coli* Fpg protein incubated with 8-OH-Gua 37mer paired opposite to C (data not shown). It should be noted, however, that GC/IDMS is less sensitive than oligonucleotide cleavage analysis and slight activity directed against a potential substrate which is revealed by sequencing gel analysis may not be detectable above background using GC/IDMS (*10*). On the basis of these results as well as the results from our previous studies (*9*, *10*) and those of Eide et al. (*5*), we conclude that a variety of methodologies indicate that 8-OH-Gua is an extremely poor substrate for Ntg1p.

DISCUSSION

The yeast *S. cerevisiae* possesses two functional homologues of *E. coli* endo III, Ntg1p and Ntg2p, although the significance of this duplication of function has remained unknown. Previous reports had speculated that these two proteins are differentially targeted within the cell (*5*, *7*, *9*). A 17 amino acid sequence at the N-terminus of Ntg1p has been proposed to be a mitochondrial signal sequence (*7*, *9*); Ntg2p possesses no such sequence. In this study, we show that Ntg1p is targeted to both the mitochondria and the nucleus, while Ntg2 is only found in the nucleus (Figure 2). Interestingly, in some cells Ntg1p is found only in the mitochondria (Figure 2A, panels E−G), while in other cells, it is localized to both the mitochondria and the nucleus (Figure 2A, panels A−C). Whether this targeting is a result of cell cycle regulation remains to be investigated. It has been shown that uracil−DNA glycosylase levels vary during the cell cycle, increasing upon DNA synthesis (*38*). It is possible that the cells possessing only mitochondrial Ntg1p may be undergoing mitochondrial DNA (mtDNA) replication or high levels of mtDNA transcription, two events which would require an increased accessibility to DNA repair proteins. Previous reports have demonstrated nuclear and mitochondrial isoforms of the same protein being encoded by a single gene (*39*, *40*), but these generally require alternative splicing or posttranslational modification of the protein. Thus far, we have not seen any evidence for modification or alternate transcriptional start sites for the production of nuclear versus mitochondrial Ntg1p, although this remains a possible mechanism for differential targeting.

We also have examined whether the differential subcellular localization of the Ntg proteins confers differential sensitivity to DNA damaging agents when either of these proteins is no longer functional. Previously, it has been shown that damage to mitochondrial DNA is more extensive and persists longer than damage to nuclear DNA (*41*). This may be due to mitochondrial DNA being more vulnerable to chemical modification due to the lack of histone association, or it may be due to a decreased repair capacity within the mitochondria. We have found that the *ntg1*, *ntg2*, and *ntg1 ntg2* mutants are no more sensitive to DNA damaging agents (including ionizing radiation, H_2O_2, menadione, and MMS) than wild-type cells (Figure 4 and data not shown), nor do they exhibit increased petite formation or mitochondrial mutation rates

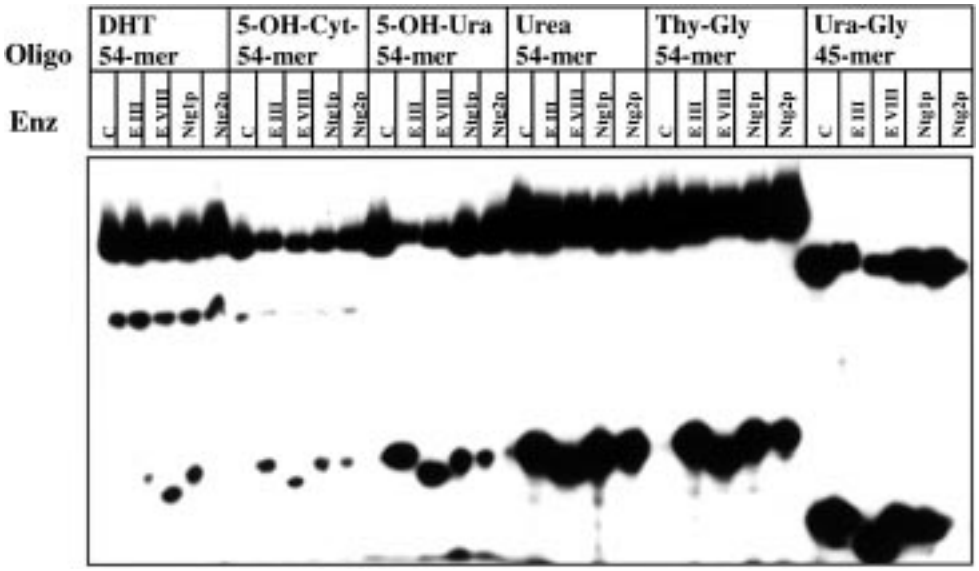

FIGURE 5: Substrate specificity of purified Ntg1p and Ntg2p. Oligonucleotides containing various DNA damages were synthesized as described in Experimental Procedures and illustrated in Table 1. Endo III, endo VIII, Ntg1p, or Ntg2p (each 100 nM) was incubated with 100 nM labeled DNA as described in Experimental Procedures. The specific oligonucleotide utilized in the reaction is indicated at the top of the figure, while the enzyme utilized is indicated above each lane [C (no enzyme), EIII (endo III), EVIII (endo VIII), Ntg1p, Ntg2p].

(spontaneously or after treatment with oxidizing agents) compared to wild-type cells (data not shown). Our findings suggest that we are either not able to specifically damage enough mtDNA molecules in order to observe a mitochondrial phenotype in *ntg1* mutants or that other repair enzymes are compensating for the loss of Ntg1p. To ascertain the biological relevance of the AP lyase activity of Ntg1p and Ntg2p, we eliminated the function of the major AP endonuclease (Apn1p) in our mutants in an attempt to reveal the extent to which AP lyases might initiate repair of abasic sites. Previous studies have shown that *E. coli* mutants lacking the two major AP endonucleases (*xth* and *nfo*) can become resistant to the killing effects of MMS when they are expressing the *Drosophila* ribosomal S3 protein, an AP lyase (*42*). We found that the *ntg1 ntg2 apn1* triple mutant was highly sensitive to the killing effects of MMS, an agent that introduces AP sites into DNA (Figure 4), demonstrating the importance of AP lyases in the removal of abasic sites. We also found that the *ntg2 apn1* mutant was more sensitive to MMS than the *ntg1 apn1* mutant. Apn1p is present exclusively in the nucleus (*43*); therefore, Ntg1p is the only known mitochondrial DNA repair protein capable of removing AP sites in *S. cerevisiae*. Due to the dispensability of mitochondrial function in yeast when provided with a fermentable carbon source (*33*), the killing effects of MMS seen in our mutant strains are most likely due to damage to the nuclear DNA. We suggest that the *ntg1 apn1* double mutant is still able to repair nuclear AP sites using Ntg2p but that the *ntg2 apn1* double mutant cannot efficiently repair nuclear DNA damage as a major proportion of Ntg1p is located in the mitochondria. The difference in survival between the *ntg1 ntg2 apn1* and *ntg2 apn1* strains reflects the contribution of Ntg1p to the removal of AP sites from nuclear DNA. Recently, a second AP endonuclease (*APN2*) was identified in *S. cerevisiae* (*44*). Interestingly, *apn2* single mutants are not sensitive to MMS, but *apn1 apn2* double mutants are highly sensitive to MMS (*44*). The subcellular localization of this protein is a question deserving further attention, but initial sequence analyses indicate Apn2p is nuclear. The effects of a combined loss of *NTG1*, *NTG2*, *APN1*, and *APN2* would presumably cause severe detriments to the cell when exposed to DNA damaging agents.

We have further demonstrated that Ntg1p and Ntg2p possess similar, but not identical, substrate specificities. In general, Ntg1p appears to recognize a wider range of damaged bases, and it also appears to better recognize certain damages when compared with Ntg2p. However, these results are based on the use of recombinant Ntg1p and/or Ntg2p, and the endogenous proteins in *S. cerevisiae* may behave differently.

We have shown the differential targeting of two important base excision repair enzymes, examined the loss of these proteins on cell survival, and further delineated the substrate specificities of these proteins. The loss of the proteins does not cause sensitivity to DNA damaging agents unless combined with a loss of other DNA repair proteins such as Apn1p. So far, *S. cerevisiae* is the only eukaryote with two endo III-like proteins which have been identified and characterized. One of these proteins, Ntg2p, possesses an iron−sulfur cluster characteristic of endo III homologues, while the other, Ntg1p, does not. The purpose of *S. cerevisiae* possessing two highly similar, but differentially localized proteins remains unknown. However, we can speculate as to possible reasons for Ntg1p being targeted to both the mitochondria and the nucleus while Ntg2p is only localized in the nucleus. The *NTG1* gene is located within a series of carbon catabolite repression proteins (*45*), which may serve as a control over the expression of mitochondrial Ntg1p under aerobic conditions (*46*) or may simply be the result of a common origin such as the mitochondrial genome. Another explanation for Ntg1p being targeted to the mitochondria in yeast could be a result of its lack of an iron−sulfur cluster. It has been shown that proteins containing iron−sulfur clusters in the mitochondria can have the iron leached out of the protein and utilized in the Fenton reaction (*47, 48*). This results in an increase in reactive oxygen species in the mitochondria and can lead to increased lipid peroxidation and free radical formation (*47, 48*). In *E. coli*, the gene for

fumarase C (*fumC*) is induced by superoxide radicals (*49*). This is the form of fumarase which lacks an iron−sulfur cluster (*49*) and could demonstrate a need for controlling the levels of iron−sulfur proteins under certain oxidative environments. While the lack of an iron−sulfur cluster in Ntg1p may not significantly alter endogenous free radical formation from the Fenton reaction, it would be logical that if an organism were given a choice between two similar repair proteins to target to the mitochondria (i.e., Ntg1p or Ntg2p), the protein with the least potential for increasing damage to DNA (Ntg1p) would be selected for mitochondrial localization. While many proteins in the mitochondria contain iron−sulfur clusters, the proximity of DNA repair enzymes to the DNA molecule may be impetus enough to avoid placing a potential hydroxyl radical generator at such a sensitive location. However, in human cells only one endo III-like protein has been identified (hNth1) (*8, 50*), and this protein is localized to both the nucleus and the mitochondria (*12*). Interestingly, the hNth1 protein is typical of endo III homologues and possesses the iron−sulfur cluster which is missing in the yeast mitochondrial endo III-like protein (Ntg1p) and has a narrower substrate specificity range compared to Ntg1p and Ntg2p. It has also been shown that another iron−sulfur cluster-containing DNA repair enzyme in human cells, hMyh (*E. coli* MutY homologue), is localized to both the nucleus and the mitochondria (*12*). However, *S. cerevisiae* does not possess a homologue to this protein, leaving the question of localization of iron−sulfur cluster DNA repair proteins to the mitochondria of yeast unanswered. The targeting of these proteins (hNth1 and hMyh) to the mitochondria in human cells despite their iron−sulfur clusters may leave human mitochondrial DNA more vulnerable to reactive oxygen species than yeast mitochondrial DNA. This may be a factor in the relationship between reactive oxygen species-induced mitochondrial DNA damage and colon cancer in humans (*4*). However, due to the redundancy seen in both *E. coli* and *S. cerevisiae* for the removal of oxidative DNA damage, the possibility of discovering a second human endo III-like protein remains.

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