

Reactive oxygen species regulate DNA copy number in isolated yeast mitochondria by triggering recombination-mediated replication

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ABSTRACT

Mitochondrial DNA (mtDNA) encodes proteins that are essential for cellular ATP production. Reactive oxygen species (ROS) are respiratory byproducts that damage mtDNA and other cellular components. In *Saccharomyces cerevisiae*, the oxidized base excision-repair enzyme Ntg1 introduces a double-stranded break (DSB) at the mtDNA replication origin *ori5*; this DSB initiates the rolling-circle mtDNA replication mediated by the homologous DNA pairing protein Mhr1. Thus, ROS may play a role in the regulation of mtDNA copy number. Here, we show that the treatment of isolated mitochondria with low concentrations of hydrogen peroxide increased mtDNA copy number in an Ntg1- and Mhr1-dependent manner. This treatment elevated the DSB levels at *ori5* of hypersuppressive [ρ^-] mtDNA only if Ntg1 was active. *In vitro* Ntg1-treatment of hypersuppressive [ρ^-] mtDNA extracted from hydrogen peroxide-treated mitochondria revealed increased oxidative modifications at *ori5* loci. We also observed that purified Ntg1 created breaks in single-stranded DNA harboring oxidized bases, and that *ori5* loci have single-stranded character. Furthermore, chronic low levels of hydrogen peroxide increased *in vivo* mtDNA copy number. We therefore propose that ROS act as a regulator of mtDNA copy number, acting through the Mhr1-dependent initiation of rolling-circle replication promoted by Ntg1-induced DSB in the single-stranded regions at *ori5*.

INTRODUCTION

Mitochondria in eukaryotic cells play a primary role in the production of ATP—the cellular source of energy—through the oxidative phosphorylation system. Mitochondria have their own genome (mtDNA), whose copy number varies in response to the physiological environment surrounding the cell (1). Genes in mtDNA, as well as in nuclear genomes, encode essential protein subunits that participate in oxidative phosphorylation. mtDNA is constantly subjected to oxidative damage due to reactive oxygen species (ROS), which are byproducts of oxidative phosphorylation. This damage results in mutations in the mtDNA, which are associated with aging and various mitochondrial disorders (2). On the other hand, recent evidence suggests that ROS regulate mtDNA replication: e.g. the treatment of human cells with hydrogen peroxide results in a transient increase in mtDNA copy number (3), and the amount of mtDNA correlates with the ROS levels in cell lines carrying different mouse mtDNA haplotypes (4). Because decreased cellular levels of mtDNA clinically correlate with mitochondrial disorders, such as mtDNA depletion syndrome, investigators are seeking to elucidate the mechanisms that regulate mtDNA copy number (5). Studies on yeast and human cells have shown that mtDNA-related nuclear gene expression and the cellular levels of deoxyribonucleoside triphosphates (dNTPs) play critical roles in the regulation of mtDNA copy number (6). Whether mitochondria contain mechanisms that regulate mtDNA copy number in response to ROS, however, is currently unknown (7).

In the yeast *Saccharomyces cerevisiae* the replication and segregation of mtDNA depend on the homologous DNA pairing protein Mhr1, which is required for mtDNA recombination (8). Generally, DNA recombination is

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initiated at a double-stranded break (DSB); after the break occurs, a homologous DNA pairing protein directs the formation of a heteroduplex joint ('the DSB repair model' of recombination) (9). Our studies revealed that the excision-repair enzyme Ntg1 creates a DSB at *ori5*, one of the active replication origins in yeast mtDNA; the DSB level increased when yeast cells were exposed to oxidative stress (10). These studies suggested that by recognizing a specific oxidative modification at *ori5*, Ntg1 induces DSB to initiate rolling-circle mtDNA replication, as well as recombination, through the homologous DNA pairing activity of Mhr1 (10). Ntg1 initiates base-excision repair of oxidative DNA damage by excising damaged bases via its DNA-N-glycosylase activity; subsequently, via its DNA lyase activity, it introduces single-stranded breaks (nicks) in the double-stranded DNA (11). Based on our previous studies showing a role for Ntg1 in the initiation of mtDNA replication, as well as a correlation between the ROS levels in mitochondria and the mtDNA copy number, we sought to uncover the molecular mechanisms underlying ROS-controlled changes in mtDNA copy number.

The Mec1/Rad53 nuclear checkpoint pathway, which is activated by nuclear DNA damage, increases mtDNA copy number by affecting the activity of ribonucleotide reductase and thereby the cellular pool of dNTPs (12). ROS are generated from either the electron transport chain or the metabolic enzyme α -ketoglutarate dehydrogenase in the tricarboxylic acid cycle; both of these ROS sources are found in normally functioning mitochondria (13). In this study, to eliminate the effects of both the Mec1/Rad53 nuclear checkpoint pathway and possible regulatory control by nuclear genes on the amounts of ROS and mtDNA, we employed isolated mitochondria harboring hypersuppressive (HS) [*ori5*] [ρ^-] mtDNA, treated with various concentrations of hydrogen peroxide, as a model system to investigate the direct effects of ROS on mtDNA copy number. Hydrogen peroxide has been used as a ROS-generating agent in a variety of studies about the effects of ROS on cellular functions.

In this study, we show that exposing isolated yeast mitochondria to hydrogen peroxide results in simultaneous increases in Ntg1-dependent DSB levels at *ori5* and in mtDNA copy number, an effect that is dependent on Ntg1 and Mhr1. It is likely that ROS trigger Ntg1- and Mhr1-dependent mtDNA replication, which leads to an increase in mtDNA copy number.

MATERIALS AND METHODS

Yeast strains

The *ntg1*-null mutant (YKN1423C-1/ Δ *ntg1*), the *mhr1*-null mutant (YKN1423C-1/ Δ *mhr1*), the *ogg1*-null mutant (YKN1423C-1/ Δ *ogg1*) cells and *cce1*-null mutant (YKN1423C-1/ Δ *cce1*) cells were derived from the YKN1423C-1 wild-type strain (nuclear genotype: α *leu2*, *ura3*, *met3*; mitochondrial genotype: HS [*ori5*] [ρ^-] mtDNA) as described previously (10). The strains WT/pVT100U and WT/pVT100UGOX were constructed by introducing pVT100U and pVT100UGOX into the

OP11c-55R5 wild-type strain (nuclear genotype: a *leu2* *ura3* *trp1*; mitochondrial genotype: [ρ^+] mtDNA). The OP11c-55R5 ρ^0 strain was constructed as described in (14).

Incubation of isolated mitochondria treated with hydrogen peroxide, the detection of ROS in isolated mitochondria, and the quantification of mtDNA levels

YKN1423C-1, YKN1423C-1/ Δ *ntg1*, YKN1423C-1/ Δ *mhr1*, YKN1423C-1/ Δ *ogg1* and YKN1423C-1/ Δ *cce1* yeast cells were cultivated to log phase in YPD medium at 30°C for 3 days. Highly enriched mitochondria were prepared and suspended in an import mixture (incubation buffer) [0.6 M mannitol, 20 mM HEPES-KOH (pH 7.4), 1 mM ATP, 1 mM MgCl₂, 5 mM phosphoenolpyruvate, 2 units of pyruvate kinase, 40 mM KCl, 5 mM methionine and 3 mg/ml BSA] as described (15), and incubated with various concentrations of hydrogen peroxide at 26°C. The mitochondria were mixed with HincII-linearized pUC119 plasmid DNA (40 μ g/ml), which was used as an internal standard. After incubation of the isolated mitochondria with or without hydrogen peroxide, DNA was extracted from the mitochondria as described previously (10). The extracted DNA was digested with BglII, which has a single recognition site in HS [*ori5*] [ρ^-] mtDNA and does not recognize pUC119 plasmid DNA. After removing proteins with proteinase K, 6 μ g of the digests were analyzed by 1.0% gel electrophoresis as described (10). A 1.0-kbp plus ladder (Invitrogen) was used as a size marker. The signals representing the HS [*ori5*] [ρ^-] mtDNA and the linearized pUC119 plasmid DNA were detected by Southern blot analysis, using ³²P-labeled HS [*ori5*] [ρ^-] mtDNA and linearized pUC119 plasmid DNA fragments as probes (16). Probe B, corresponding to the *ori5* region, was amplified with primers Probe B-F (5'-GTTATATATTT ATATATTTTC-3') and Probe B-R (5'-CTTTTATTTT TATTCTAT-3'). Probe C, corresponding to the segment outside the *ori5* region (0.28 kbp) was amplified with the Probe C-F (5'-ATAAGAATTTAATAAGTTAT-3') and Probe C-R (5'-TCTAGAGGATCTTCTTCATTATA-3'). The ³²P signals were qualitatively and quantitatively analyzed with a Fuji BAS2500 image analyzer as described previously (10), and the relative mtDNA copy number was calculated by normalizing the signals for HS [*ori5*] [ρ^-] to those for linearized pUC119.

Detection of newly synthesized mtDNA

Isolated mitochondria were suspended in 100 μ l of incubation buffer containing 0.1 mM dATP, 0.1 mM dCTP, 0.1 mM dGTP, 0.065 mM dTTP and 0.035 mM DIG-11-dUTP (Roche) and incubated at 26°C. Aliquots (20 μ l) were taken at various time points for mtDNA extraction. Southern dot-blotting analysis was performed using Hybond-N⁺ membrane as described previously (16). The signals representing newly synthesized mtDNA were detected with anti-DIG-AP conjugates and a DIG detection kit (Roche). Ethidium bromide (EtBr; 10 μ g/ml) was used to inhibit mtDNA replication (17).

Assay for oxidative modifications at *ori5* by detecting increased DSB levels induced *in vitro* by purified Ntg1

The standard reaction mixture (12 μ l) consisted of 15 μ g of BglII-digested DNA, 70 mM 3-(*N*-morpholino)-propane-sulfonic acid (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol and 1.6 nM purified Ntg1 protein, as previously described (10). The reaction was carried out at 37°C for 0.5 h as described (11). After proteins were removed with proteinase K, DNA digests electrophoresed on a 2.0% agarose gel at room temperature for 24 h. The signals representing the 0.8-kbp DNA fragment derived from DSBs at *ori5* in HS [*ori5*] [ρ^-] mtDNA digested with BglII and 1.1-kbp unit length of HS [*ori5*] [ρ^-] mtDNA were detected by Southern blot analysis using ³²P-labeled 1.1-kbp HS [*ori5*] [ρ^-] mtDNA as a probe (10).

Assay for Ntg1-mediated nicking in dsDNA in an *ori5*-containing region of HS [*ori5*] [ρ^-] mtDNA

Isolated mitochondria from wild-type cells were mixed with HincII-linearized pUC119 plasmid DNA (40 μ g/ml), which was used as an internal standard. After incubation of the isolated mitochondria with or without hydrogen peroxide at 26°C for 1 h, DNA was extracted from the mitochondria as described previously (10). The BglII–NdeI digested DNA was treated with purified Ntg1 protein (1.6 nM) in the standard reaction mixture at 37°C for 30 min, as described above. After the removal of proteins with proteinase K, the DNA digests were mixed with alkaline loading buffer containing 50 mM NaOH, 1 mM EDTA, 3% Ficoll (w/v), 0.025% bromocresol green (3,3',5,5'-tetrabromo-*m*-cresolsulfonphthalein) (w/v) and 0.004% (w/v) xylene cyanol and were then loaded onto a 2.0% alkaline agarose gel. Denaturing agarose gel electrophoresis was performed in 50 mM NaOH and 1 mM EDTA at 30 V/cm for 15 h as described in (18). The signals representing the denatured HS [*ori5*] [ρ^-] mtDNA and pUC119 plasmid DNA were detected by Southern blot analysis using ³²P-labeled HS [*ori5*] [ρ^-] mtDNA and linearized pUC119 plasmid DNA fragments as probes (16).

Assay for the S1-nuclease sensitivity of HS [*ori5*] [ρ^-] mtDNA

HS [*ori5*] [ρ^-] mtDNA, extracted from isolated mitochondria derived from wild-type cells, was digested with BglII and NdeI. Subsequent treatment with S1 nuclease was performed in a standard reaction mixture (12 μ l) consisting of 3 μ g of BglII–NdeI digested DNA, 30 mM sodium acetate buffer (pH 4.6), 280 mM NaCl, 1 mM ZnSO₄ and various amounts of S1 nuclease at 37°C for 20 min. Digests were separated by electrophoresis on a 2.0% agarose gel. Signals were detected by Southern blot analysis using ³²P-labeled 1.1-kbp HS [*ori5*] [ρ^-] mtDNA as a probe.

Assay for breakage at an oxidized base in single-stranded DNA by purified Ntg1

The 30-mer oligonucleotides containing either single oxidized base, 5-hydroxyuracil (5-OHU) or thymine at the

14th position (5'-CTCGTCAGCATCTXCATCATAACA GTCAGTG-3') were used as the single-stranded DNA substrates. These oligos were synthesized and purified by Tsukuba Oligo Service and labeled using the DIG 3'-end labeling kit (Roche). The reaction was performed in a buffer suitable for Ntg1 activity as described in (5), which contained various amounts of purified Ntg1 or C Δ ntg1 as indicated in Figure 5F, at 37°C for 30 min. The reaction was stopped by the addition of an equal volume of 90% formamide loading dye solution. The samples were heated at 75°C for 2 min and run on a 20% polyacrylamide gel containing 7 M urea. The signals representing each oligo were detected using the DIG detection kit (Roche).

Detection of *GOX* expression and assays for relative mtDNA copy number and hydrogen peroxide levels

The *GOX* open reading frame was amplified by PCR from *Aspergillus niger* as described in (19). The *GOX* gene was subcloned into the pVT100U vector (20) at a blunted HindIII/XhoI site, where it is under control of the *ADH* promoter. WT cells harboring pVT100U or pVT100UGOX were cultivated in a 2.0% glucose medium (SD) supplemented with required amino acids at 30°C and harvested before the glucose in the medium is completely consumed. The cell-free extracts were prepared as described in (21). *GOX* expression was confirmed in the WT cells harboring pVT100UGOX by Western blot analysis using a monoclonal antibody against glucose oxidase (Sigma). As a control, the mitochondrial outer membrane protein porin was detected using a monoclonal anti-porin antibody (molecular probes).

Hydrogen peroxide levels in cells with or without expression of *GOX* were detected using a Fluoro hydrogen peroxide detection kit (Cell Technology Inc.), after cells suspended in PBS buffer containing 0.5-mm glass beads were disrupted using a Beads Shocker (Yasui Kikai). The amount of fluorescent product resorufin generated from the chemical reaction in which hydrogen peroxide oxidizes 10-acetyl-3,7-dihydroxyphenoxazine was measured at excitation 570 nm and emission 595 nm in fluorescent plate reader (Molecular devices).

Whole cellular DNA was extracted as described in (10). About 20 μ g DNA was separated by electrophoresis on a 1.0% agarose gel. [ρ^+] mtDNA was detected by Southern analysis using highly purified total mtDNA as a probe. *NUC1* was detected on the same Southern blot using PCR-amplified DNA fragments of the *NUC1* open reading frame as a probe.

RESULTS

Increases in mtDNA copy number in isolated mitochondria harboring HS [*ori5*] [ρ^-] mtDNA following the treatment with hydrogen peroxide

Hydrogen peroxide is widely used as an oxidant to investigate the effects of ROS on cellular functions (22). To examine the effects of ROS on mtDNA replication, isolated yeast mitochondria harboring HS [*ori5*] [ρ^-] mtDNA were treated with various concentrations of

hydrogen peroxide in the incubation buffer. The signals representing mtDNA were detected using Southern analysis with a 282-bp DNA fragment from the *ori5* region and a 280-bp DNA fragment outside of the *ori5* region, in addition to full-length HS [*ori5*] [ρ^-] mtDNA (Figure 1A). As examples, increased and decreased levels of mtDNA detected using full-length HS [*ori5*] [ρ^-] mtDNA as a probe are shown in Figure 1B. The relative mtDNA copy number was calculated from the signal from HS [*ori5*] [ρ^-] mtDNA relative to that of the extrinsic standard, HincII-linearized pUC119 plasmid. The amount of DNA increased in response to treatment with hydrogen peroxide up to 10 μ M (Figure 1C). No significant difference was detected between the relative levels of mtDNA measured using the *ori5*-specific probe, the probe located outside of *ori5*, or the full-length probe, when isolated mitochondria were treated with hydrogen peroxide concentrations ranging from 0 to 100 μ M (Figure 1C, inserts). This is not a peculiar feature of HS [*ori5*] [ρ^-] mtDNA. We have reproduced the major results of the ROS-induced increase in mtDNA copy number using isolated mitochondria containing [ρ^+] mtDNA (Supplementary Figure 1).

On the other hand, as suggested by previous observations that highly reactive oxygen radicals can damage various cell structures, including mitochondria (23), higher concentrations of hydrogen peroxide (≥ 20 μ M) appeared to rupture the isolated mitochondria, leading to a gradual increase in amount of eluted proteins (Figure 1D); no increase was observed after treatment with ≥ 20 μ M hydrogen peroxide (Figure 1B and C). A higher concentration of hydrogen peroxide may also inhibit enzymes required for mtDNA replication; likewise, an elevated level of oxidative mtDNA damage may slow the progression of mtDNA replication.

No detectable increases in mtDNA copy number were observed in mitochondria isolated from *ntg1*-null or *mhr1*-null mutant cells treated with hydrogen peroxide up to 10 μ M. In contrast, disruption of the *OGG1* gene, which encodes another excision repair enzyme that recognizes 8-oxoguanine, did not suppress the increases in mtDNA copy number (Figure 1B and C). Thus, mtDNA copy number increases in a manner dependent on the functions of *Ntg1* and *Mhr1*.

Previously, we showed that the maintenance of yeast mtDNA depends on the recombination-related genes, *MHR1* and *CCE1* (14). Nuclear *CCE1* gene encodes a mitochondria-localized recombination junction-resolving endonuclease, and likely plays a role in mtDNA recombination (24,25). In response to treatment with hydrogen peroxide up to 10 μ M, the amount of DNA in mitochondria isolated from *ccel*-null mutant cells increased and was only slightly less than that observed in mitochondria isolated from wild-type cells (Figure 1B and C). Thus, the *Cce1*-dependent recombination system is a minor pathway for the hydrogen peroxide-induced increase in mtDNA copy number.

Treating isolated mitochondria with 10 μ M hydrogen peroxide resulted in an increase in mtDNA copy number (Figure 2A), which doubled in the first 0.5 h of incubation (Figure 2B). The rate of this increase slowed after 1 h of incubation (Figure 2B). Since the addition of fresh

hydrogen peroxide (10 μ M) after 1 h induced a further increase in mtDNA copy number (up to 4.5-fold in another 1 h; data not shown), it is likely that hydrogen peroxide underwent decomposition during incubation.

Without hydrogen peroxide treatment, mtDNA copy number in mitochondria isolated from wild-type cells increased only after a lag period of 0.5 h (Figure 2A and B). The difference in the copy number between treated and untreated mitochondria was about 2-fold after 0.5 h of incubation (Figure 2B). Vitamin C, vitamin E and *N*-acetylcysteine (NAC) are widely used antioxidants that eliminate ROS (26,27). No increase in mtDNA copy number was observed, when mitochondria were incubated with 500 μ M vitamin C, 25 μ M vitamin E, or 500 μ M *N*-acetylcysteine from the start time point (Figure 2B). In addition, the increase in mtDNA copy number was suppressed by transferring the hydrogen peroxide-untreated mitochondria into fresh incubation buffer containing 500 μ M vitamin C after 1 h of incubation (Figure 2B). Thus, it is likely that the increase in mtDNA copy number was induced even in isolated mitochondria not treated with hydrogen peroxide due to ROS generated during the incubation. Isolated mitochondrial α -ketoglutarate dehydrogenase and pyruvate dehydrogenase complexes generate ROS (13). It is likely that ROS generated from these complexes eventually activate mtDNA replication in isolated mitochondria. These observations showed that the increases in mtDNA copy number observed in isolated mitochondria with or without hydrogen peroxide treatment were caused by increased levels of ROS (Figure 2B). In the following experiments, we examined the first 1 h of the incubation, in which the effects of the added hydrogen peroxide is prominent.

Next, we investigated whether the hydrogen-peroxide-induced increase in mtDNA copy number was a result of a net increase in newly synthesized mtDNA. We proceeded by examining digoxigenin (DIG)-11-dUTP-labeled mtDNA in isolated mitochondria, whose levels reflect incorporation of the labeled nucleotide throughout the mtDNA (Figure 2C, left panel). As a control, the same amount of DNA extracted from isolated mitochondria at various reaction time points was spotted onto a Hybond- N^+ membrane. The signals derived from total mtDNA were detected by Southern dot-blotting analysis using 32 P-labeled 1.1-kbp HS [*ori5*] [ρ^-] mtDNA as the probe (Figure 2C, right panel). It has been shown that isolated yeast mitochondria incorporate dNTPs into mtDNA (17). The incorporation of DIG-dUTP into mtDNA of isolated mitochondria was observed for 1–1.5 h after the addition of the labeled dNTP, an effect that was enhanced by 10 μ M hydrogen peroxide (Figure 2C, left panel). DIG-dUTP-derived signals were not observed in isolated mitochondria in the presence of 10 μ g/ml EtBr—an inhibitor of mtDNA polymerase γ —indicating that the increased signals reflected mtDNA synthesis (Figure 2C). Consistent with the preceding experiment, isolated mitochondria treated with a higher concentration of hydrogen peroxide (100 μ M) did not incorporate DIG-dUTP into mtDNA (Figure 2C).

In mitochondria isolated from *ntg1*-null or *mhr1*-null mutant cells, increases in mtDNA copy number were not

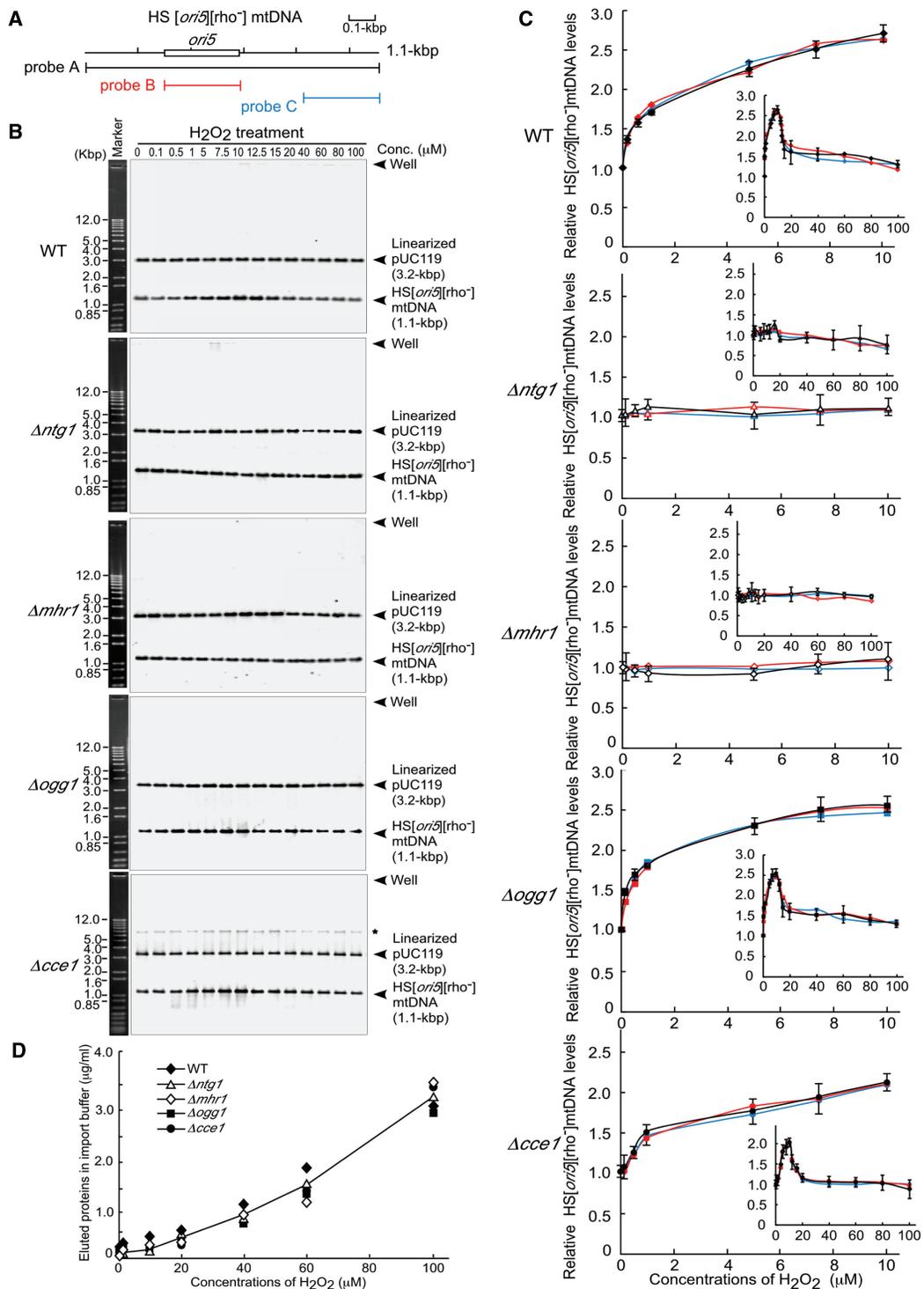


Figure 1. mtDNA copy number in isolated mitochondria harboring HS [*ori5*][*rho*⁻] mtDNA, which were treated with various concentrations of hydrogen peroxide. (A) A physical map of the locations of the *ori5*-region and the probes used for detecting HS [*ori5*][*rho*⁻] mtDNA. Probe A, full length HS [*ori5*][*rho*⁻] mtDNA (1.1 kbp); probe B, a 282-bp region of *ori5* and probe C, a 280-bp sequence outside of the *ori5* region. The *ori5* sequence (282 bp) is framed with an open box. (B) HS [*ori5*][*rho*⁻] mtDNA-containing mitochondria derived from wild-type, *ntg1*-null, *mhr1*-null, *ogg1*-null and *cce1*-null cells were suspended in incubation buffer and incubated with the indicated concentrations of hydrogen peroxide for 1 h at 26°C. HS [*ori5*][*rho*⁻] mtDNA and linearized pUC119 plasmid DNA were separated on a 1.0% agarose gel and detected by Southern blot analysis using the sequences described in (A) and linearized pUC119 as probes. Marker, 1.0-kb plus ladder. Asterisk denotes mtDNA molecules containing single-stranded regions resistant to digestion with BglIII, likely to be a Holliday junction structure, since they mainly appeared in HS [*ori5*][*rho*⁻] mtDNA extracted from Holliday junction resolvase-lacking *cce1*-null cells. (C) Quantitative analysis of the mtDNA bands in (B). Upper panel: wild type (filled diamonds); upper middle panel: *ntg1*-null mutant (open triangles); lower middle panel: *mhr1*-null mutant (open diamonds); bottom panel: *ogg1*-null mutant (filled squares) and lower bottom panel: *cce1*-null mutant (filled circles). In each panel, the colored lines representing the relative mtDNA copy number detected using Southern analysis with various probes. Black lines, probe A; red lines, probe B and blue lines, probe C. Inset in (C), a figure showing the increase of the HS [*ori5*][*rho*⁻] mtDNA copy number in isolated mitochondria treated with hydrogen peroxide concentrations ranging from 0 to 100 μ M. (D) Eluted proteins from inside isolated mitochondria, accompanied by treatment with hydrogen peroxide.

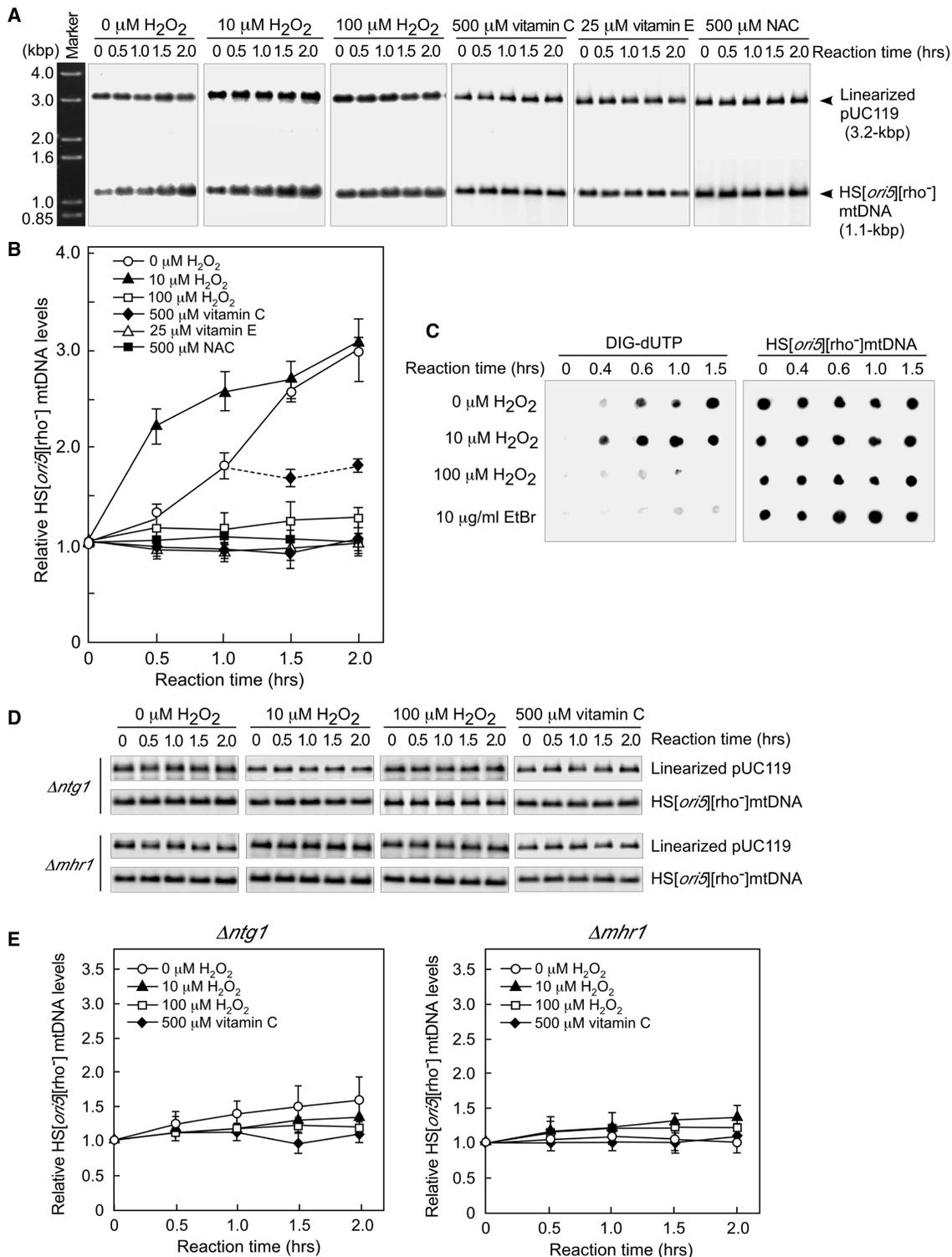


Figure 2. Changes in mtDNA copy number in isolated mitochondria treated with 10 μM hydrogen peroxide. (A) Treatment with 10 μM hydrogen peroxide increased mtDNA copy number in mitochondria isolated from wild-type cells. Isolated mitochondria were incubated with the indicated concentrations of hydrogen peroxide. mtDNA was digested with BglIII and separated by electrophoresis on a 1.0% agarose gel. Marker, 1.0-kbp plus ladder. (B) Quantitative analysis of the relative mtDNA copy number in mitochondria isolated from wild-type cells. Vitamin C (500 μM), 25 μM vitamin E or 500 μM NAC were added to the incubation buffer from the start time point. After 1 h of incubation, mitochondria not treated with hydrogen peroxide were transferred into fresh incubation buffer containing 500 μM vitamin C. The average values from three independent experiments are plotted in (B). (C) Newly synthesized mtDNA (left panel) and total mtDNA (right panel) in isolated mitochondria. Mitochondria isolated from wild-type cells were incubated with DIG-dUTP, which was detected using anti-DIG AP conjugates. Total mtDNA was detected by Southern dot-blotting analysis using ³²P-labeled 1.1-kbp HS[ori β][rho⁻] mtDNA as the probe. (D) A low concentration (10 μM) of hydrogen peroxide did not induce an increase in mtDNA copy number in mitochondria isolated from either *ntg1*-null or *mhr1*-null cells. There was no change in mtDNA copy number when mitochondria isolated from either *ntg1*-null or *mhr1*-null cells were incubated with vitamin C. (E) Quantitative analysis of mtDNA copy number in mitochondria isolated from either *ntg1*-null or *mhr1*-null mutant cells. The average values from three independent experiments are plotted.

observed during a 2-h incubation, regardless of whether the samples were treated with 10 μ M hydrogen peroxide, or were incubated in the presence of antioxidants (Figure 2D and E). Furthermore, we did not observe a significant change in mtDNA copy number in isolated mitochondria containing [rho⁺] mtDNA derived from *ntg1*-null or *mhr1-1* cells (Supplementary Figure 1D and E). These results indicate that the increase in mtDNA copy number requires both Ntg1 and Mhr1.

Increased DSB levels at *ori5* in hydrogen peroxide-treated mitochondria

We previously showed that Ntg1-induced DSBs initiate mtDNA replication (10). Therefore, we investigated whether the levels of DSBs at *ori5* in HS [*ori5*] [rho⁻] mtDNA correlated with changes in mtDNA copy number induced by hydrogen peroxide treatment. The 0.8-kbp DNA fragment corresponded to the fragment between the DSB site at *ori5* and the BglII site (10). As shown in Figure 3A and B, the increased DSB level correlated with the increase in mtDNA copy number: in mitochondria isolated from wild-type cells, the DSB levels at *ori5* increased during 1 h of incubation in 10 μ M hydrogen peroxide, but did not change during a 1 h incubation without hydrogen peroxide (Figure 3A and B). The number of DSB at *ori5* gradually decreased when hydrogen peroxide-untreated mitochondria were incubated with antioxidants (vitamin C, vitamin E or NAC), suggesting the DSB levels at *ori5* are associated with the ROS levels (Figure 3A and B). The addition of antioxidants did not affect mtDNA copy number (Figure 2), suggesting that the content of mtDNA was sustained at a basal level.

In mitochondria isolated from *ntg1*-null mutant cells, the DSB levels at *ori5* were about half of those observed in wild-type cells, independent of the hydrogen peroxide treatment or the presence of vitamin C and remained constant during a 1-h incubation (Figure 3C and D); thus, Ntg1 plays an important role in DSB formation at *ori5*. A slight increase in the DSB levels was observed in 10 μ M hydrogen peroxide-treated mitochondria isolated from *ntg1*-null mutant cells. These data, along with the basal DSB levels at *ori5* in mtDNA derived from *ntg1*-null mutant cells, indicate that there is another mechanism that introduces DSBs at *ori5* (12). The Ntg1-independent DSBs may be formed by an unidentified functional homolog of Ntg1, or by the action of an unidentified mitochondrial S1 nuclease-like enzyme that cuts single-stranded structures in double-stranded DNA to create single-strand DNA breaks; during replication, these breaks would then be converted into DSBs in the *ori5* region.

The DSB levels at *ori5* in HS [*ori5*] [rho⁻] mtDNA from hydrogen peroxide-treated mitochondria isolated from *mhr1*-null mutant cells increased during 1 h of incubation, just as those from *MHR1* cells did (Figure 3E and F versus 3A and B). In HS [*ori5*] [rho⁻] mtDNA from hydrogen peroxide-untreated *MHR1* cells, DSB levels did not change during 1 h of incubation. In contrast, DSB levels increased in HS [*ori5*] [rho⁻] mtDNA isolated from hydrogen peroxide-untreated *mhr1*-null mutant cells; furthermore, the increase in DSB was suppressed by vitamin C

(Figure 3E and F versus 3A and B). This difference likely reflects Mhr1-dependent recombination functions such as recombinational DSB repair (9).

Accumulation of oxidative modifications at *ori5* that are recognized by Ntg1

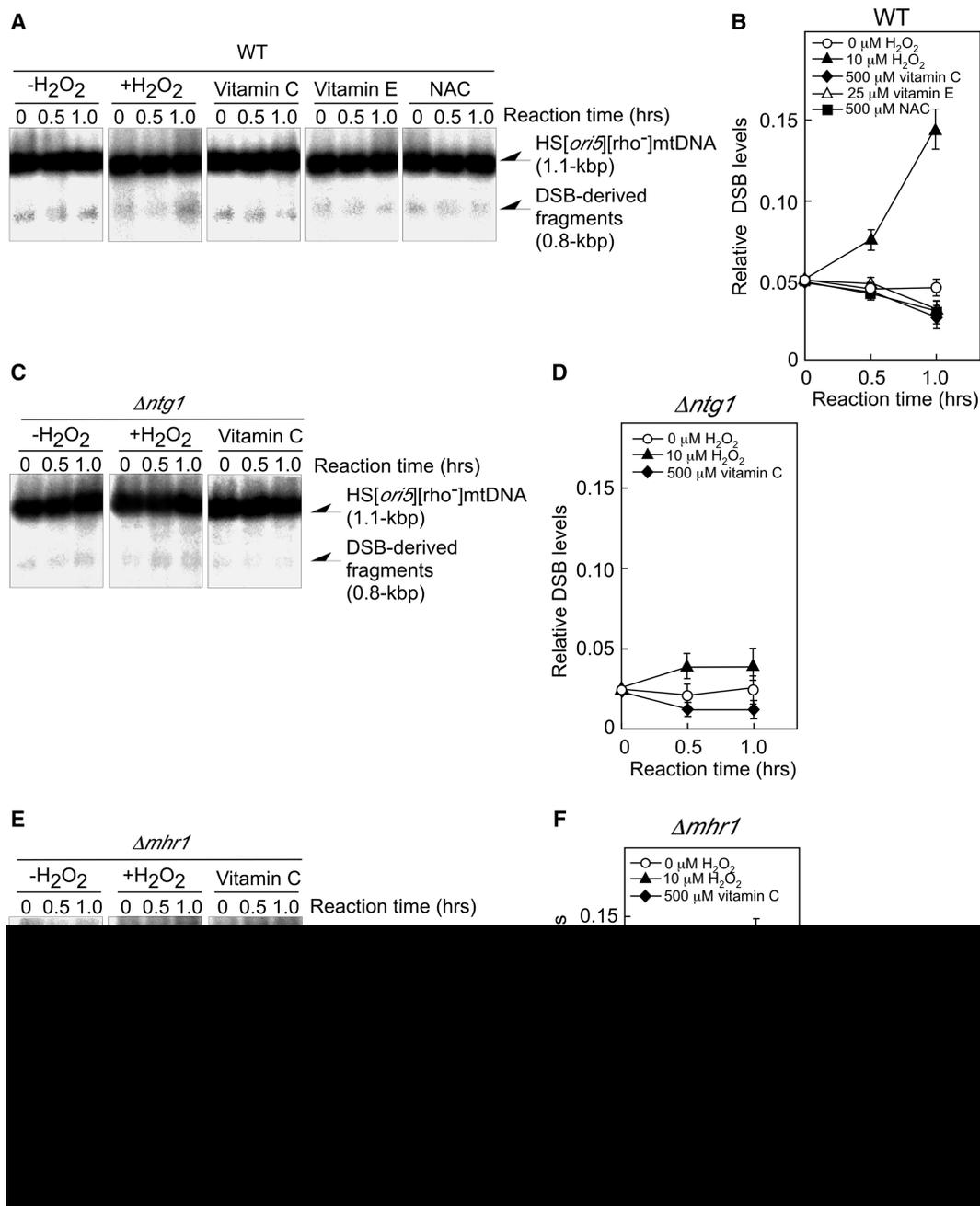
To obtain direct evidence that hydrogen peroxide treatment enhances *ori5*-specific oxidative modifications of mtDNA in isolated mitochondria, we measured the DSB levels at *ori5* in HS [*ori5*] [rho⁻] mtDNA, as an indication of the unrepaired oxidative damage that accumulated throughout the mtDNA (10). We treated DNA purified from the isolated mitochondria with purified Ntg1, as described previously (10). The resulting increases in the DSB levels served as a measure of the levels of oxidative modifications at *ori5*. The DSB levels at *ori5* in HS [*ori5*] [rho⁻] mtDNA purified from untreated mitochondria isolated from wild-type cells did not change during a 0.5-h incubation (Figure 4A, lane 1 versus lane 3), whereas the DSB levels increased slightly (1.3-fold) due to Ntg1 treatment (Figure 4A, lane 1 versus lane 2 and lane 3 versus lane 4). The DSB levels at *ori5* in mtDNA isolated from hydrogen peroxide-treated mitochondria doubled following Ntg1 treatment for 0.5 h (Figure 4A, lane 5 versus lane 6), indicating that oxidative modifications accumulated at *ori5* as a result of hydrogen peroxide treatment.

In agreement with previous results that showed Mhr1 is required for the repair of spontaneously introduced oxidative damage in yeast mtDNA (28), we found that the DSB levels induced by Ntg1 treatment *in vitro* (i.e. oxidative damage at *ori5*) were higher (3-fold) in mtDNA purified from hydrogen peroxide-treated mitochondria derived from *mhr1*-null mutant cells, as compared with the results obtained using *MHR1* cells (2-fold) (Figure 4B, lanes 5 and 6, versus Figure 4A, lanes 5 and 6). Thus, treating isolated mitochondria with hydrogen peroxide causes an increase in the number of oxidative modifications at *ori5*; furthermore, Mhr1 is involved in repair of the DSBs induced at *ori5* by Ntg1.

Is there an unusual structure underlying the DSBs introduced by Ntg1 at *ori5*?

Ntg1 is a DNA *N*-glycosylase/AP-lyase, which has been shown to remove oxidative bases in double-stranded DNA and to introduce single-stranded breaks in the strand with the abasic site generated (11,29). The known activities of Ntg1 cannot explain the observation that it can also introduce DSBs by itself only in the *ori5*-region, but not in the rest of HS [*ori5*] [rho⁻] mtDNA (10). Ogg1, which specifically recognizes the oxidation-damaged guanine bases (30), did not induce DSBs in the *ori5*-region (10). DSBs introduced by DNA *N*-glycosylase/AP-lyase are formed when two or more lesions are very closely positioned in opposite DNA strands to form clustered lesions (31). Thus, we addressed the question of how Ntg1 creates the DSBs and how DSB occurs specifically at *ori5*, with respect to both the activity of Ntg1 and the structure of the *ori5* region.

Since we assumed that *ori5*-region is more sensitive to oxidative modification by ROS, we first asked whether



that Ntg1 introduced a larger number of nicks in the *ori5*-region than in other regions. HS [*ori5*] [*rho*⁻] mtDNA and the internal standard, HincII-linearized pUC119 plasmid DNA from isolated mitochondria treated or untreated with hydrogen peroxide, were incubated with or without purified Ntg1 *in vitro*. BglIII-NdeI DNA-digests were

loaded onto a 2.0% alkaline agarose gel, which was run at a sufficiently high pH to denature double-stranded DNA. After digestion with BglIII and NdeI HS [*ori5*] [*rho*⁻], mtDNA gave rise to a 0.74-kbp *ori5*-containing and a 0.38-kbp *ori5*-lacking DNA fragments (Figure 5B, bottom panel: Southern blot). The ratio of the