Spontaneous DNA Damage in Saccharomyces cerevisiae Elicits Phenotypic Properties Similar to Cancer Cells*

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To determine the spectrum of effects elicited by specific levels of spontaneous DNA damage, a series of isogenic Saccharomyces cerevisiae strains defective in base excision repair (BER) and nucleotide excision repair (NER) were analyzed. In log phase of growth, when compared with wild type (WT) or NER-defective cells, BER-defective cells and BER/NER-defective cells possess elevated levels of unrepairable, spontaneous oxidative DNA damage. This system allowed establishment of a range of ~400 to 1400 Ntg1p-recognized DNA lesions per genome necessary to provoke profound biological changes similar in many respects to the phenotypic properties of cancer cells. The BER/NER-defective cells are genetically unstable, exhibiting mutator and hyper-recombinogenic phenotypes. They also exhibit aberrations in morphology, DNA content, and growth characteristics compared with WT, BER-defective, and NER-defective cells. The BER/NER-defective cells also possess increased levels of intracellular reactive oxygen species, activate the yeast checkpoint response pathway via Rad53p phosphorylation in stationary phase, and show profound changes in transcription patterns, a subset of which can be ascribed to responses resulting from unrepairable DNA damage. By establishing a relationship between specific levels of spontaneous DNA damage and the ensuing deleterious biological consequences, these yeast DNA excision repair-defective strains are an informative model for gauging the progressive biological consequences of spontaneous DNA damage accumulation and may have relevancy for delineating underlying mechanisms in tumorigenesis.

Extensive spontaneous DNA damage occurs continuously through normal metabolic processes. Such damage includes base loss, oxidative damage, deamination, and alkylation. Of these, the two most prevalent classes of endogenous DNA lesions are thought to be depurination, which results in noncoding abasic sites, and oxidative damage, which results in strand breaks, base modifications, abasic sites, and DNA-protein cross-links (1). Accordingly, it is estimated that 10,000 oxidative hits occur per cell per day in the mammalian genome (2). To maintain genomic integrity, cells have evolved a number of mechanisms to either repair or tolerate DNA damage. In Saccharomyces cerevisiae, these pathways include direct reversal, base excision repair (BER),1 nucleotide excision repair (NER), mismatch repair (MMR), translesion synthesis, and recombination (3). In mammalian systems, inability to properly repair DNA damage or respond to its presence can result in a number of deleterious biological consequences, including cell death, mutation, and neoplastic transformation (4).

BER involves primarily the repair of small, helix non-distorting base lesions and abasic sites (5). It is believed that repair of oxidative damage is mediated primarily by this pathway (6). In NER, the DNA damage is recognized by a specific DNA glycosylase that catalyzes the release of the damaged base and creates an abasic site. In S. cerevisiae, the abasic site can be processed further by either a hydrolytic AP endonuclease such as Apn1p or N-glycosylase-associated AP lyases such as Ntg1p or Ntg2p. DNA strand cleavage resulting from the actions of these proteins results in modified strand break termini that must be further processed to become substrates for subsequent repair proteins such as DNA polymerase and DNA ligase (7).

In contrast to BER, NER is believed to repair bulky, helix-distorting lesions such as bipyrimidine UV photoproducts (5). However, several reports indicate that there is considerable overlap between the DNA excision repair pathways, BER and NER, with respect to damage processing. For example, similar to BER, NER has been shown to be capable of processing oxidative lesions such as thymine glycol and 8-oxoguanine (8–10). In addition, it was demonstrated that both BER and NER repair abasic sites (11, 12). More recently, synergism between BER and NER for the repair of endogenous and exogenous oxidative DNA damage was demonstrated (13).

When excision repair is compromised, it could be expected that a large proportion of spontaneous DNA damage will be left unrepaired and cause deleterious biological consequences. However, in DNA repair defective yeast cells, the level of spontaneous DNA damage existing in the genome has neither been determined nor connected with specific biological endpoints. DNA damage has been implicated in the development of cancer, aging, and other degenerative conditions (14). Although the role of unrepaired DNA damage in mutagenesis and the initiation of carcinogenesis in mammalian cells is well established, its contribution to the progression of tumor develop-

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1 The abbreviations used are: BER, base excision repair; NER, nucleotide excision repair; MMR, mismatch repair; DAPI, diaminophenylindole; FACS, fluorescence-activated cell sorting; ROS, reactive oxygen species; ORF, open reading frame.

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ment and the acquisition of a genetic instability phenotype is poorly understood, particularly with respect to the levels of DNA damage required to drive these processes (4).

We have employed a series of haploid yeast mutants defective in BER (ntg1 ntg2 apn1), NER (rad1), or BER and NER (ntg1 ntg2 apn1 rad1) to determine the effects of spontaneous DNA damage on cellular processes in S. cerevisiae. As shown previously, it is necessary to disrupt APN1, NTG1, and NTG2 to severely compromise the BER pathway, because single knockouts or double knockout combinations of these three genes do not exhibit obvious phenotypic abnormalities (11). Our results demonstrate that cells acquire remarkably high levels of spontaneous DNA damage that are normally repaired by BER and NER. In addition, we show that, when excision repair is compromised, unrepaird spontaneous DNA damage, upon reaching specific levels, results in gross morphological abnormalities, genetic instability, a chronic stress state characterized by high intracellular ROS levels, profound changes in transcription patterns, and changes in Rad53 phosphorylation. These features are similar to those observed in cancer cells, suggesting that yeast can be used as an informative model for assessing the progressive biological consequences of unrepaired DNA damage in higher eukaryotes and the underlying mechanisms leading to such changes.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Growth Conditions**—All strains used in this study are isogenic derivatives of SJR751 (MATa ade2–101 his3200 ura3 Ako lys2 Bgl2 leu2–1). SJR751 will be referred to as wild type (WT). BER-defective strain SJR867 (MATa ade2–101 his3200 ura3 Ako lys2 Bgl2 leu2–1 niu1::LEU2 ntu2::hisG apn1::HIS3), NER-defective strain SJR868 (MATa ade2–101 his3200 ura3 Ako lys2 Bgl2 leu2–1 niu1::LEU2 rad1::hisG), and BER/NER-defective strain SJR1101 (MATa ade2–101 his3200 ura3 Ako lys2 Bgl2 leu2–1 niu1::LEU2 ntu2::hisG apn1::HIS3 rad1::hisG). Isogenic strains were constructed as described previously (11).

We previously observed that the BER/NER-defective strain consistently produced a mixture of large and small colonies when grown on rich YPD medium (11). When these colonies were microscopically examined, small colonies were found to be composed entirely of large cells nearly twice the size of WT cells, whereas large colonies were composed of a mixture of large cells as well as those that were similar in size to WT (Fig. 2A). Therefore, the BER/NER-defective strain was subdivided into two morphological population types, BER/NER-defective mixed cell type or BER/NER-defective large cell type, representing mixed cell size and large cell size populations, respectively. These designations refer to the cell composition of independent colonies from BER/NER-defective strains used to inoculate experimental cultures.

Yeast strains were grown on YPD medium (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar for plates). All YPD media was supplemented with adenine sulfate (US Biological) at the recombinant concentration with a single yeast colony and grown at 30 °C for 24 h and then viewed at 100 × magnification. For mutagenesis and recombination studies, YNB media supplemented with required amino acids was utilized (2% glucose, 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, and 2% agar). Supplemented YNB medium lacking lysine was used for selective growth of Lys+ recombinants and revertants. Supplemented YNB medium lacking arginine and containing 60 mM sodium citrate, the cutoff for the DAPI stain used was used for selective growth of canavanine-resistant (CAN) mutants.

**Cell Growth, Viability, and Microscopy**—YPD medium was inoculated with a single yeast colony and grown at 30 °C to a density of 1 × 10^9 cells/ml. An aliquot was used to inoculate fresh YPD medium to a density of 1 × 10^9 cells/ml. After 15 h of growth, cells were counted and diluted to a final cell density determined. For microscopic analysis, YPD medium was inoculated with a single colony, and cells were grown for 96 h. Cells were harvested and resuspended in fresh YPD medium 48 and 72 h after initial inoculation. 96 h after inoculation, aliquots were streaked onto YPD plates. Plates were placed at 30 °C for 24 h and then viewed at 10× magnification. Approximately 300 of the streaked colonies were counted as viable (formed colony of greater than five cells) or non-viable (failed to form colony of greater than five cells). For microscopy, 1-ml aliquots of cells were harvested 12, 48, 72, and 96 h following initial inoculation. Cells were resuspended in 70% ethanol and 1 μl of 1 mg/ml dianisomethyleninde (DAPI) was added. Samples were viewed by microscopy at 100× magnification in both phase contrast and after DAPI staining. For all morphological studies, cells were sonicated prior to viewing to ensure that clumps of cells, composed of distinct cell units, were not mistaken for extensively branched single unit networks.

**DNA Isolation and Processing**—Cells were grown in YPD for 24 h (log phase of growth) or 72 h (stationary phase). After 24 or 72 h of growth, ~2 × 10^9 cells were harvested by centrifugation and resuspended in 0.8 ml of sorbitol EDTA (0.9 mM sorbitol, 0.1 mM EDTA, pH 7.4) containing 25 μl of dithiothreitol (1 mM) and 100 μl of zymolyase 20T (10 mg/ml) (US Biologicals). Cells were incubated at 37 °C overnight. Spheroplasts were spun down, resuspended in 0.5 ml of Tris-EDTA (50 mM Tris-HCl, pH 7.4, 20 mM EDTA) containing 50 μl of 20 mg/ml proteinase K (Boehringer Manheim), and incubated at 55 °C for 1 h. In 1 ml of 100 mM sodium acetate (5 M), samples were incubated on ice for 30 min. Samples were centrifuged, supernatants were transferred, and two chloroform extractions were performed. DNA was precipitated with isopropanol, resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and digested with 5 μl of ribonuclease A (10 mg/ml) (US Biologicals) at 37 °C for 2 h. Samples were precipitated with ethanol, washed twice, lyophilized, and resuspended in TE buffer. To isolate DNA from cells exposed to H_2O_2, the same protocol was utilized with the following alterations: 2 × 10^9 cells were harvested by centrifugation, washed once in sterile water, and resuspended to the initial volume with sterile water. Cells were treated with 2.5 mM H_2O_2 (Sigma) for 30 min at 30 °C and then harvested by centrifugation and washed twice with sterile water. This DNA extraction procedure was designed to minimize the level of adventitious DNA damage. DNA was quantified by fluorometry using the fluorescent dye bis-benzimide (Hoechst 33258) (Sigma DNA Quantitation kit, DNA-QP). Fluorometry was performed according to the manufacturer’s recommendations. 10-μg aliquots of yeast DNA were digested with BamH1 and ApaL1 (New England Biolabs), using 2.5–3 units of each enzyme per microgram of DNA, for 1 h at 37 °C. Digestion with these enzymes yields a 3.7-kb fragment containing the 1.8-kb coding sequence of the CAN1 gene and a 1.9 kb of flanking sequence on chromosome V. After restriction, DNA was precipitated with ethanol, washed twice, lyophilized, and resuspended at a concentration of ~1 μg/μl. DNA concentration of the digested samples was determined by the average of at least five independent fluorometric readings.

**Alkaline Gel Electrophoresis**—Gel electrophoresis was performed using a 1.1% alkaline agarose gel in alkaline electrophoresis buffer (30 mM NaOH, 2 mM EDTA, pH 8) for 20 min at 40 V and then for 15 h at 17 °C (17). Genomic DNA samples were prepared as follows. 10 μg of BamH1- and ApaL1-digested genomic DNA was incubated with 5 μg of recombinant glutathione S-transferase-tagged NTG1 protein in a 25-μl reaction mixture containing buffer B (15 mM KHPO_4, pH 6.8, 10 mM EDTA, 10 μg H_2O_2, and 40 μl RCI) (6). All sample volumes were kept constant, and reactions were incubated at 37 °C for 30 min. To stop the reaction, samples were heated at 60 °C for 5 min. An internal standard corresponding to a 170-bp segment of the CAN1 gene, generated by PCR amplification, was added to each sample, after Ntg1p incubation, at a concentration of 0.15 ng/μg of genomic DNA. Prior to loading, denaturing dye (50 mM NaOH, 1 mM EDTA, pH 8) was added to each sample. The recombinant Ntg1p used in these experiments is N-terminally tagged with glutathione S-transferase and expressed in Escherichia coli as described previously (6). The protein was purified by glutathione-agarose chromatography followed by Mono-S fast-performance liquid chromatography (18).

**Transfer of DNA to Membranes and Hybridization**—Transfer and hybridization were performed according to Sambrook and Russell (19), with the following modifications. Transfer was performed utilizing alkaline transfer buffer (20× SSC, 0.4 mM NaOH). After 24 h of transfer, DNA was UV-cross-linked to the nylon membrane and pre-hybridized at 65 °C in rapid-hyb buffer (Amersham Biosciences). For hybridization, a 423-bp PCR-generated fragment of the CAN1 gene was used as the template for PCR labeling and probe preparation. The 3.7-kb CAN1 gene fragment was determined by PhosphorImager analysis of the Southern blot and used to determine levels of oxidative DNA damage. All CAN1 fragment bands were normalized to corresponding internal standard bands to control for sample loading variations. The Poisson expression was used to calculate the number of oxidative DNA damage events. The DAPI stain was visualized by PAG gel electrophoresis as described previously (15, 16). The primary Rad53 antibody was used from Santa Cruz Biotechnology.
Biotechnology. For phosphatase treatments, the trichloroacetic acid precipitate was washed twice in acetone, resuspended in phosphate buffer (Roche Applied Science) and incubated for 30 min at 37 °C with 10 units of calf intestine phosphatase (Roche Applied Science).

Measurement of Spontaneous Mutation and Recombination Frequencies—YPD media was inoculated with a single colony and cells were grown to saturation. With an aliquot of this culture, fresh YPD media was inoculated to a density allowing harvest of ~2 × 10^6 cells/ml after 15 h of growth. To assess Can™ mutations and Lys™ recombinants, cells were harvested and plated as described (11). To identify lys®ΔBgl revertants, the following modifications were made. Cell aliquots were plated onto complete YNB medium to determine initial viability. The remaining cells were resuspended in YPD media and allowed to grow for 1 h at 30 °C. Cells were then harvested and plated onto complete YNBD medium to determine viable cell number in the outgrowth population. Remaining cells were plated onto YNB medium lacking lysine to select for Lys™ revertants. The lys®ΔBgl mutation frequency values were corrected for the number of generations undergone during outgrowth. Both Can™ and Lys™ colonies were counted 3 days after selective plating. The mutation and recombination frequencies reported in Table II were calculated as follows: the number of mutants or recombinants/total cell number. Potential jackpot cultures were identified as outliers statistically by using the Grubbs test (21). These values were withheld from frequency calculations presented in Table II.

The data from a minimum of 20 independent cultures was used for each mutation and recombination frequency determination.

Flow Cytometry—3 ml of cells at ~5 × 10^6 cells/ml was centrifuged for 5 min at 3020 rpm. The supernatant was removed, and cells were resuspended in 1 ml of water. Cells were centrifuged again, and the supernatant was removed. The resulting pellet was resuspended in 1 ml of absolute ethanol and stored at 4 °C overnight. Cells were vortexed vigorously, centrifuged, resuspended in 1 ml of water, and pelleted again. The pellet was then resuspended with 1 ml of 50 mM sodium citrate (pH 7.0) and transferred to appropriate FACS analysis tubes (Falcon 2054, 12 × 75 mm). 8 μl of 10 mg/ml DNase-free RNase A was added, and samples were incubated for 1 h at 50 °C. 25 μl of 10 mg/ml of proteinase K was added, and the samples were incubated for 1 h at 50 °C. The samples were then stained with 1 μl of propidium iodide (16 μg/ml) in 50 mM sodium citrate (pH 7.0). Samples were shielded from light and analyzed using a BD Biosciences FACSCalibur® and CellQuest® software.

Reactive Oxygen Species Levels—ROS levels were measured with dihydrodihoramide 123 according to Qi et al. (22), with the following modifications. 5 ml of mid-log phase cell culture was treated with 2.5 μg/ml dihydrodihoramide 123 for 2 h at 30 °C. The cell culture was washed and resuspended in PBS and analyzed using a FACSCalibur® and CellQuest® software. Wild type cells were exposed to 3 or 4 mM H_2O_2 or left untreated for 30 min at 30 °C. Cells were washed once, resuspended in media, and then treated with dihydrodihoramide 123 for ROS assessment.

Microarray Analysis of Gene Expression—Cells were monitored for growth until they reached ~5 × 10^6 cells/ml and were exposed to 0.75 mM H_2O_2 or left untreated for 30 min at 30 °C. Following treatment, cells were harvested and total RNA was isolated. Total RNA was extracted from cells using the hot-phenol method. RNA was then purified on an RNA affinity resin (RNeasy Mini Kit, Qiagen, Chatsworth, CA). The total RNA was ethanol-precipitated and resuspended in 15 μl of diethyl pyrocarbonate-treated water (RNase-free). Total RNA was quantitated by UV spectrophotometry. Approximately, 20 μg of total RNA was used in the cDNA synthesis reaction. The cDNA synthesis procedure was carried out as described in the Affymetrix GeneChip Expression Analysis Technical Manual, using a modified oligo(dT) primer with a T7 RNA polymerase primer on the 5' end of the sequence (5'-GGCCAGTGAAATTGTAATACGACTCACTATAGGG-AGGCGG-(dT)24-3'). cDNA was ethanol-precipitated and resuspended in 12 μl of diethyl pyrocarbonate water. 3.3 μl of cDNA was used for the cDNA biotin-labeling reaction. cDNA was labeled with Bio-11-CTP and Bio-16-CTP as detected in the ENZAX protocol. High Yield RNA transcript labeling kit (ENZO Diagnostics, Farmingdale, NY). cDNA was purified on an affinity resin (RNeasy Mini Kit, Qiagen) and was fragmented as described in the Affymetrix GeneChip Expression Analysis Technical Manual. Fragmented cDNA was quantitated by measuring absorbance at 260 nm, and purity was ascertained by determining the absorbance ratio A_260nm/A_280nm. All RNA samples yielded ratios of 1.9 or greater indicating appropriate RNA quality.

Chip Hybridization and Data Analysis—The hybridization mix was heated at 99 °C for 5 min and then 45 °C for 5 min. Following a 5-min centrifugation, 200 μl of the hybridization mixture was loaded to YSG98 microarray chips (Affymetrix, Santa Clara, CA) containing ~6400 ORFs.c. O. Following hybridization, the chips were loaded into the fluidics station for washing and staining as described in Section 2, Chapter 4 of the Affymetrix GeneChip Expression Analysis Technical Manual. After staining and washing, the chips were loaded into the GeneArray Scanner and scanned using a specialized confocal laser scanning microscope and analyzed using Microarray Suite version 5.0. The arrays were scaled to have a target intensity of 150 in order for all chips to be compared with one another. Integrity of RNA samples was determined by comparing the signal intensity of probes derived from the 5'-end and the 3'-end of actin and the TATA-binding protein. The signal ratio 5' to 3' did not exceed 2.5-fold, indicating that the mRNA was not degraded during preparation. For all chips, Affymetrix Statistical Algorithms were applied in the analysis of the probe arrays as described in the Statistical Algorithms Reference Guide (obtainable from Affymetrix, Santa Clara, CA, available at www.affymetrix.com). Expression information can be found on the Doetsch laboratory website (www.biochem.emory.edu/labs/medpwd/microarray_index.html).

RESULTS

Spontaneous Oxidative DNA Damage Accumulates When Excision Repair Is Compromised.—To investigate the effects of unrepair, spontaneous oxidative DNA damage on various biological and genetic endpoints, we first determined the relative levels of nuclear oxidative DNA damage in the WT, BER-defective, NER-defective, and BER/NER-defective strains via a gene-specific damage-detection assay. The BER/NER-defective strain was analyzed as two distinct morphological population types, BER/NER-defective mixed cell type and BER/NER-defective large cell type (see “Experimental Procedures”). DNA was extracted from each of the strains after 24 h (log phase) or 72 h (stationary phase) of growth and subjected to restriction digestion to yield a 3.7-kb fragment containing the 1.8-kb coding sequence of the CAN1 gene and 1.9 kb of flanking sequence on chromosome V. The CAN1 locus was assessed for DNA damage, because this same region was also used to obtain spontaneous mutation frequencies in these strains. The restricted genomic DNA was then subjected to treatment with Ntg1p, an N-glycosylase with associated AP lyase activity that primarily recognizes and cleaves DNA containing oxidative pyrimidine base damage and abasic sites (6). The resulting digests were analyzed by denaturing agarose gel electrophoresis. Undamaged DNA (not cleaved by Ntg1p) will yield an
intact 3.7-kb band. DNA containing oxidative pyrimidine base damage and abasic sites within this 3.7-kb sequence will be cleaved, resulting in a decrease in the level of the 3.7-kb band as revealed by Southern blot analysis (23).

Genomic DNA from WT- and NER-defective cells in log phase of growth revealed no reduction of the 3.7-kb CAN1 gene segment following incubation with Ntg1p, whereas DNA from BER-defective cells showed a modest reduction (Fig. 1 and Table I). In contrast, genomic DNA extracted from both BER/NER-defective mixed and large type cells in log phase of growth contained significant levels of unrepaired oxidative damage recognized by Ntg1p, resulting in a reduction of ~23 and 35% in the CAN1 signal, respectively (Fig. 1 and Table I). Using the Poisson formula to estimate DNA damage, the frequency of Ntg1p-recognized DNA lesions per 3.7-kb CAN1 gene fragment was calculated (20). The number of lesions per fragment was negligible in WT cells, 0.26 in BER/NER-defective mixed type cells, and 0.43 in BER/NER-defective large type cells, corresponding to the presence of 840 and 1400 Ntg1p-recognized lesions per genome, respectively (Table I). The level of oxidative base damage induced in WT cells by an acute exposure to \( \text{H}_2\text{O}_2 \) (2.5 mM), resulting in 58% killing, corresponds to 1360 Ntg1p-recognized lesions per genome (Fig. 1 and Table I). After 72 h of growth (stationary phase), an increase in Ntg1p-recognized DNA lesions was observed for all strains (Table I). This result is consistent with the observation that more ROS are present in stationary phase yeast cells (24).

The finding that the BER/NER-defective strain contained such high levels of genomic oxidative DNA damage is remarkable and indicates that these cells are capable of tolerating substantial, chronic spontaneous DNA damage. In addition, these measurements allow for the connection of a particular level of unrepaired DNA damage with associated deleterious biological consequences as described below.

**Presence of Spontaneous DNA Damage Adversely Affects Cell Growth, Viability, and Morphology**—Initial microscopic examination of the BER/NER-defective cells indicated that both mixed and large type cells possess large proportions of morphological variants. These variants were characterized by defining, for cultures of all strains, the frequencies (percentages) of the following morphological types: normal shaped (N), abnormally shaped/single bud (A-S), normal shaped/multiple buds (N-M), and abnormally shaped/multiple buds (A-M) (Fig. 2A). Total abnormal morphology was defined as the summed percentage of morphological types A-S, N-M, and A-M. Following 96 h of continuous growth in rich media, BER/NER-defective cells, mixed cell type and large cell type, exhibited 10 and 13% total abnormal morphology, respectively (Fig. 2D). In contrast, WT, BER-defective, and NER-defective cells exhibited 5% or less total abnormal morphology. Furthermore, although WT, BER-defective, and NER-defective cells exhibited negligible abnormal morphologies after 12 h of growth, BER/NER-defective mixed and large type cells display 5 and 8% abnormal morphologies, respectively (Fig. 2C). For all strains, the percentage of cells with abnormal morphology increases with the duration of growth. However, in the case of the BER/NER-defective strain, the appearance of cells with abnormal morphologies occurs at an earlier stage of growth and rapidly accelerates (Fig. 2C). The gross appearance of colonies in the BER/NER-defective strain is also abnormal (Fig. 2B). When cells were grown for 96 h in rich media, plated, and examined by microscopy following 24 h of growth, colony edges were ragged in contrast to the smooth appearance of WT (Fig. 2B), BER-defective, and NER-defective colonies (not shown). Moreover, such ragged colony edges often extended in branched protrusions from the central colony mass, reminiscent of filamentous growth induced by environmental stress or slowed DNA synthesis (27, 28).

In addition to displaying abnormal cellular and colony morphology, BER/NER-defective cells (both mixed cell type and large cell type) grew at ~50% (180 min per generation) of the rate of WT cells (data not shown). Because the growth rates of the BER/NER-defective strain types were substantially retarded, the viability of this strain after 96 h of growth in rich media was determined. WT, BER-defective, and NER-defective cells exhibited less than a 5% decrease in viability over 96 h of continuous growth, whereas BER/NER-defective cells showed a marked decrease in viability, with mixed cell type and large cell type exhibiting decreases of 20 and 40%, respectively (data not shown). Such a decrease in viable, colony-forming cells can be observed in microscopic fields containing large numbers of single, non-viable BER/NER-defective cells (Fig. 2B). Thus, adverse effects on cellular and colony morphology, cell growth, and viability accompany deficiencies in excision repair.

**Increased DNA Content in Excision Repair-defective Cells**—The observed slow growth and aberrant cellular morphology, especially the large cell size, of the BER/NER-defective strain could reflect abnormalities in cell cycle progression. To address whether BER/NER-defective cells possess altered cell cycle properties, cells in log phase of growth were subjected to FACS analysis. WT, BER-defective, and NER-defective cells show a normal distribution of G1 and G2/M cells as evidenced by two peaks (Fig. 3A). The profile for the BER/NER-defective strain, however, shows a profound shift and broadening of these two peaks. Such a peak profile is similar to those that have been previously shown to correspond to cells with increased DNA content and size (29). The DNA content profile and the large cell size of the BER/NER-defective large cell type suggest that this strain possesses a 2N or greater DNA content at all times. This was confirmed by isolating DNA from equal numbers of WT, BER-defective, NER-defective, and BER/NER-defective

### Table I

| Strain                          | Ratio of band intensities of Ntg1p treated to untreated samples | Lesions per 3.7kb CAN1 fragment | Lesions per genome |
|---------------------------------|---------------------------------------------------------------|-------------------------------|-------------------|
|                                 | 24 h             | 72 h             | 24 h | 72 h | 24 h | 72 h |
| WT                              | 1.0 ± 0.030      | 0.84 ± 0.043     | 0    | 0.17 | 0    | 550  |
| BER-defective                   | 0.89 ± 0.081     | 0.70 ± 0.054     | 0.12 | 0.36 | 380  | 1170 |
| NER-defective                   | 1.0 ± 0.060      | 0.78 ± 0.028     | 0    | 0.25 | 0    | 810  |
| BER/NER-defective mixed cell type | 0.77 ± 0.039     | ND               | 0.26 | ND   | 840  | ND   |
| BER/NER-defective large cell type | 0.65 ± 0.044     | 0.56 ± 0.032     | 0.43 | 0.58 | 1400 | 1880 |
| WT \( \text{H}_2\text{O}_2 \)  | 0.66 ± 0.051     | ND               | 0.42 | ND   | 1360 | ND   |
|                                 | **Lesions calculated using the Poisson expression: \( S = -\ln P_c \) (S, lesions per 3.7-kb CAN1 fragment; \( P_c \), ratio of band intensities of Ntg1p-treated to untreated samples).** |
|                                 | **Values extrapolated from calculation of lesions per 3.7-kb CAN1 fragment.** |
|                                 | **After 24 and 72 h of growth, cells were in log phase and stationary phase, respectively.** |
|                                 | **ND, not determined.** |
mixed and large type cells. The DNA content per cell of the BER/NER-defective large cell type was significantly higher (2.5- to 4-fold) than that of the other strains (Fig. 3B). Attempts to monitor cell cycle progression were unsuccessful because of the exquisite sensitivity of the BER/NER-defective cell types to the toxic effects of nocodazole and hydroxyurea (data not shown). However, the sensitivity of the BER/NER-defective strain to these cell cycle checkpoint drugs indicates that these cells may not respond properly to checkpoint controls, regardless of which stage of the cell cycle these inhibitors are acting upon (30).

**Presence of Spontaneous DNA Damage Triggers Checkpoint Signaling in Stationary Phase**—Phosphorylation and activation of the Rad53p kinase are important indicators of the activation of DNA damage and replicational stress checkpoints that initiate cell cycle arrest, mediate transcriptional responses, and enhance DNA repair (25, 26). When Rad53p modification was detected in cell extracts and monitored as a function of growth stage of untreated cultures, significant phosphorylation was observed only in BER/NER-defective cells and only upon entry into stationary phase (2 days) (Fig. 4).

**Excision Repair Defects Confer a Genetic Instability Phenotype**—To investigate the effect of the excision repair defect on genome stability, spontaneous mutation and recombination frequencies were determined. Two types of spontaneous mutation frequencies were monitored: forward mutations at the CAN1 locus and reversion events of the lys2/H9004Bgl frameshift allele. Forward mutations in the arginine permease gene CAN1 render the locus non-functional and the cells resistant to the toxic arginine analog L-canavanine (31). A 3N-1 frameshift mutation within a 150-bp reversion window in the lys2/H9004Bgl allele yields Lys/H11001 prototrophs (32). For recombination measurements, recombination between two non-functional LYS2 alleles, lys2ΔBgl on chromosome II and lys2Δ3500 on chromosome V, was measured by Lys+ prototroph production (33). Previous studies revealed significant increases in both spontaneous mutation and recombination rates of the BER/NER-defective strain over WT in stationary (non-dividing) phase (11). However, because abnormal cellular morphology was first observed during log phase of growth in the BER/NER-defective strain, but not the WT, BER-defective, or NER-defective strain, mutation and recombination frequency status during log phase

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**Fig. 2.** Cell and colony morphology of DNA repair pathway defective strains. A, cellular morphology of WT, BER-defective, NER-defective, BER/NER-defective mixed and large type cells in log phase of growth. Cells were examined by microscopy in phase contrast (top panel) and after DAPI staining (bottom panel). Morphological types: normal shaped (N), abnormally shaped/single bud (A-S), normal shaped/multiple buds (N-M), abnormally shaped/multiple buds (A-M). The same magnification (100×) was used for all strains. Bars, 10 μm. B, bright field microscopy of WT and BER/NER-defective colonies. Bar (bottom, right), 200 μm. C, total abnormal morphological types found in WT (diamonds), BER-defective (squares), NER-defective (triangles), BER/NER-defective mixed cell type (circles), and BER/NER-defective large cell type (asterisks) strains after 12, 48, 72, and 96 h of continuous growth in supplemented YPD medium. D, contributions of abnormal morphological sub-types (black, A-M; hashed, N-M; white, A) to total abnormal morphology of WT, BER-defective, NER-defective, BER/NER-defective mixed cell type and BER/NER-defective large cell type after 96 h of continuous growth in supplemented YPD medium.
of growth was determined. Both mutation and recombination frequencies were moderately elevated in the BER-defective strain compared with the WT and NER-defective strains (Table II). In contrast, when BER and NER are simultaneously compromised, mutation and recombination frequencies increase synergistically. The BER/NER-defective strain was increased 56- and 48-fold over WT in CanR and <sup>lys2</sup>/<sup>H9004</sup> Bgl mutation frequencies, respectively, and 121-fold above WT in recombination frequency. Such increases in mutation and recombination frequencies suggest that when DNA excision repair is compromised, the remaining unrepaired, spontaneous DNA damage is accompanied by increases in at least two types of genetic instability.

**Elevated ROS in Excision Repair-defective Cells**—ROS are produced through normal cellular metabolic processes or exposure to stress agents and are thought to be important for signal transduction pathways associated with control of transcription and cell growth (34, 35). To determine whether unrepaired spontaneous DNA damage leads to an increase in ROS, log phase cells were grown in the presence of dihydrorhodamine, an intracellular ROS indicator (22). The WT, BER-defective, and NER-defective strains have identical profiles for intracellular ROS content, whereas the BER/NER-defective strain has 29% (mixed cell type) and 56% (large cell type) of cells with substantially higher intracellular ROS levels compared with the other strains (Fig. 5A). These results indicate that unrepaired spontaneous DNA damage leads to an increase in intracellular ROS. In addition, the BER/NER-defective large cell type ROS profile is intermediate to that produced by exposures to 3 and 4 mM H₂O₂ in the WT strain (Fig. 5B). These results indicate that unrepaired spontaneous DNA damage leads to an increase in intracellular ROS. In addition, the BER/NER-defective strain has adapted to survive under conditions of oxidative stress, which, when given to WT cells as an acute exposure to H₂O₂, produce similar intracellular ROS levels that are accompanied by a high degree of toxicity.
increasing levels of intracellular ROS. Genes are up-regulated (2- to 289-fold), and 13 genes are down-regulated (elevated 4.5-fold), identified as being induced by a number of known DNA damaging agents (37–39). Specifically, 122 genes or without exposure to H2O2. WT, unexposed (blue exposure (green)). When the wild type strain was exposed to a dose of H2O2, WT, unexposed (black); WT, 3 mM H2O2 exposure (green); WT, 4 mM H2O2 exposure (pink); BER/NER-defective large cell type, unexposed (blue); x-axis, from left to right, represents increasing levels of intracellular ROS.

Global Gene Expression Profiles in DNA Repair-proficient and -deficient Strains—To evaluate the impact of defects in the BER and/or NER pathways on patterns of gene expression, the transcription profiles of WT, BER-defective, NER-defective, and BER/NER-defective large type cells were determined by gene microarray analysis. The gene expression pattern of WT cells was compared with itself (WT), BER-defective, NER-defective, or BER/NER-defective cells (Fig. 6). The genes are represented on scatter plots by red dots and are positioned according to the signal in one strain compared with the signal in the other strain. Dot linearity represents similarity in signal intensity between strains, indicating little or no change in gene expression. In the BER/NER-defective strain, the gene expression pattern varied significantly from the WT strain (Fig. 6D). This pattern was not observed for the strains defective in either BER or NER alone (Fig. 6, B and C). When the wild type strain was exposed to a dose of H2O2 resulting in ~50% killing, the expression pattern also varied significantly from the untreated WT expression pattern (Fig. 6E). There was ~21% overlap in the genes up-regulated in the BER/NER-defective large type cells compared with WT cells exposed to a toxic dose of H2O2. Such genes include UBI4 (elevated 3.3-fold), encoding ubiquitin, which has been shown to be necessary for regulation of DNA repair activities (36), and GPM2 (elevated 4.5-fold), identified as being induced by a number of known DNA damaging agents (37–39). Specifically, 122 genes are up-regulated (2- to 289-fold), and 13 genes are down-regulated (2- to 254-fold) in the BER/NER-defective large type cells when compared with WT cells (no H2O2 exposure) (data represent three independent experiments). All expression data information can be found at www.biochem.emory.edu/labs/medpwd/microarray_index.html.

The above results suggest that unrepaired, spontaneous DNA damage in the BER/NER-defective cells results in DNA damage-signaling responses that alter their gene expression patterns. For example, RNR3 and HUG1, known responders to DNA damage, are up-regulated in BER/NER-defective cells (elevated 6.2- and 21.5-fold, respectively), but not in WT cells. When cells are defective for BER or NER, the remaining DNA damage-handling pathways are able to process spontaneous DNA damage in such a way that does not result in this DNA damage-induced response. The genes up-regulated in the BER/NER-defective large type cells, when compared with WT cells, can be organized into a number of functional groups, including protein degradation, metabolism, mitochondrial maintenance, DNA replication/repair, and cell wall maintenance. 60 of these 122 up-regulated genes represent unknown ORFs. Two of these up-regulated genes, also unknown ORFs, are essential for viability.

DISCUSSION

Because spontaneous DNA damage occurs as a consequence of normal cellular processes, multiple DNA damage-handling pathways are essential. Although such pathways have been shown to act cooperatively in response to DNA damage, it is logical to assume that, under conditions of compromised excision repair, with only DNA damage tolerance mechanisms such as translesion synthesis and recombination remaining functional, the majority of spontaneous DNA damage will remain in the genome and exert various deleterious effects. However, to our knowledge, this is the first report that establishes a relationship between specific genomic levels of un repaired, spontaneous DNA damage, and the resulting biological and genetic consequences.

In log phase of growth (24 h), we have shown that WT and NER-defective cells have negligible levels of Ntg1p-recognized lesions. This finding is somewhat surprising considering that NER repairs a number of spontaneously occurring Ntg1p-recognized lesions (8–12). In contrast, BER-defective cells possess moderately elevated levels of DNA damage, ~400 Ntg1p-recognized lesions per genome (Table I). Such unrepair DNA damage in the BER-defective strain results in no obvious deleterious effects with respect to growth, viability, morphology, cell cycle, intracellular ROS, and global gene expression patterns but does cause a moderate increase in mutation and recombination frequencies (Table II). Therefore, spontaneous levels of DNA in the range of ~400 Ntg1p-recognized lesions per genome cause genetic instability but no other observable deleterious biological consequences.

In contrast to the single pathway-defective strains, BER/NER-defective cells in log phase of growth (24 h) have dramatically elevated levels of DNA damage (~1400 Ntg1p-recognized lesions per genome) and consequently exhibit profound biolog-
critical and genetic changes. The BER/NER-defective strain displays characteristics similar to cancer cells, such as genetic instability, abnormal growth, morphological aberrations, increased levels of intracellular ROS, and altered global transcription profiles (4). Significantly, these results establish a range of greater than 400 but less than 1400 Ntg1p-recognized lesions per genome necessary to provoke adverse biological effects. However, because Ntg1p recognizes only a subset of DNA lesions resulting from spontaneous processes, our measured DNA damage frequencies represent an underestimate of the total number of lesions present per genome. Our system does, however, demonstrate that as spontaneous DNA damage accumulates there is a progression of deleterious consequences beginning with genetic instability followed by other biological abnormalities. Levels of spontaneous DNA damage, in the range of 400 lesions per genome, result in genetic instability, but no other biological consequences. However, when spontaneous DNA damage levels reach ~1400 lesions per genome, a constellation of additional biological changes occur.

Despite the significantly elevated spontaneous DNA damage levels in the BER/NER-defective strain in log phase of growth, activation of the Rad53p DNA damage checkpoint pathway does not occur. However, when BER/NER-defective cells enter stationary phase, DNA damage levels increase and Rad53p becomes phosphorylated. This suggests that Rad53p phosphorylation status is dependent upon the amount of unrepaired oxidative DNA damage present in cells. This also suggests that there is a narrow threshold of DNA damage that needs to be exceeded for Rad53p checkpoint activation (in our study, between 1400 and 1900 Ntg1p-recognized lesions per genome). Because Rad53p is autocatalytic, this supports our observation that a narrow threshold of DNA damage is needed for its activation (40). In addition, checkpoint activation in stationary phase (non-dividing) cultures suggests that unrepaired DNA damage triggers checkpoint activation rather than interference with replication or a DNA repair intermediate, although we cannot rule out a contribution from recombination repair. Similar observations have been made for UV-induced DNA damage (15). In humans, repair-proficient senescent cell cultures are characterized by phosphorylated Chk2 (the human Rad53p homolog), presumably due to telomere erosion (41). However, our results indicate that oxidative DNA damage may also play a role in regulation of senescence. The absence of Rad53p phosphorylation during the growth phase of a culture could reflect a lower DNA damage level. Alternatively, the absence of Rad53p phosphorylation could represent a need to actively dampen the DNA damage signal to allow for replication in the presence of unrepaired DNA damage. This process, known as adaptation, is clearly correlated with reduction of Rad53p phosphorylation in budding yeast (42). A silenced checkpoint system could also provide an explanation for our inability to synchronize the BER/NER-defective cells with the checkpoint-triggering agents nocodazole and hydroxyurea.

Because the only difference between the WT and the BER/NER-defective strain is in the ability to repair spontaneous DNA damage, global genome expression analysis allowed identification of responses resulting specifically from unrepaired, spontaneous DNA damage present in the genome of BER/NER-defective cells. Because Rad53p phosphorylation was not detected in BER/NER-defective cells in log phase of growth, these DNA damage-dependent changes in transcription do not involve the Rad53p-dependent DNA damage checkpoint pathway. However, the up-regulation of RNR3 and HUG1 in BER/NER-defective cells supports the idea that DNA damage signaling is occurring. For example, RNR3 is maintained at low levels in wild type yeast and is induced only upon signaling resulting from DNA damage (43). Signaling in response to DNA damage is dependent on Mec1p (44), which suppresses mec1 lethality (suggesting HUG1 is a negative regulator of MEC1) is induced following activation of Mec1p (45). In our study, significant up-regulation of HUG1, a 20-fold increase over WT, was observed in BER/NER-defective cells. Consequently, to allow for survival, we speculate that HUG1 may be highly up-regulated to suppress the continuous DNA damage signal/response occurring in BER/NER-defective cells. Furthermore, 64% of the genes up-regulated in the BER/NER-defective cells

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**Fig. 6. Comparative gene expression patterns in WT and DNA repair pathway defective strains.** Scatter plots of mRNA signals from WT cells compared with (A) WT, (B) BER-defective cells, (C) NER-defective cells, (D) BER/NER-defective cells, and (E) WT cells exposed to an acute dose of H2O2 (0.75 mM). Each dot represents the signal intensity of one mRNA species. Dots are positioned according to the signal intensity in one strain compared with the signal intensity in the other strain. Diagonal lines represent 2, 5, 10, and 30-fold differences in transcript levels.
have been identified in previously published transcriptional studies as being up-regulated by exposure to exogenous DNA damaging agents, including methylmethane sulfonate, ionizing radiation, and H₂O₂ (37–39). These studies support the notion that many changes in expression in the BER/NER-defective cells are due to responses to unrepaired, spontaneous DNA damage. Therefore, the 122 genes identified in this study not only represent candidate responders to DNA damage but may also represent genes that are necessary for survival and growth under conditions of chronic oxidative stress caused by DNA damage. As a result, further analysis of these strains, under conditions of acute exposure to DNA-damaging agents, may be useful for identifying genes responding specifically to the presence of DNA damage as opposed to other signaling pathways initiated outside of the nucleus.

Our findings also show that unrepaired, spontaneous DNA damage in BER/NER-defective cells results in increased levels of intracellular ROS. It has been observed that changes in ROS levels are accompanied by changes in signal transduction pathways controlling transcription and cell growth (34, 35). The increased levels of ROS in the BER/NER-defective cells may indicate changes in signal transduction pathways involved in yeast cell survival when DNA damage levels are chronically elevated and left unrepaired. In human cells, it has been observed that, upon p53 pathway activation, which occurs in response to DNA damage, levels of ROS increase (35). The increased ROS levels may also indicate changes in the regulation of proteins through post-translational modifications necessary for repair of DNA damage. For example, RNR3, which is a component of ribonucleotide reductase, is regulated by its redox state. To produce dNTPs, which are necessary for repair and to execute DNA repair or damage tolerance, ribonucleotide reductase activity is up-regulated through redox signaling during S-phase and in response to DNA damage (43).

In the BER/NER-defective cells, the normal cellular and colony morphologies, due to unrepaired, spontaneous DNA damage, are reminiscent of environmental stress-induced pseudohyphal growth and the morphologies of cells with mutations in cell cycle regulators CDC28 and cyclins (27, 46). These morphologies are also consistent with those initiated by MEC1 in response to slowed DNA synthesis in haploid yeast (28). Although the above study failed to show induction of filamentous growth by sublethal doses of DNA-damaging agents, our results reveal that the accumulation of unrepaired, spontaneous DNA damage elicits a similar filamentous phenotype. These similarities, as well as the documented growth delays, cell size enlargement, and increased DNA content of the BER/NER-defective cells, suggest that these cells possess cell cycle abnormalities. This idea is further supported by their extreme sensitivity to DNA damage checkpoint drugs. However, because Rad53p phosphorylation was detected in BER/ NER-defective cells in stationary phase, this suggests that in mid-log phase of growth, either the Rad53p checkpoint pathway is silenced or another cell cycle checkpoint pathway is corrupted. Together, these results indicate that acquisition of specific levels of unrepaired, spontaneous DNA damage not only results in genetic instability but also profoundly affects global gene expression patterns, intracellular ROS levels, and cell cycle properties. Below these levels of DNA damage, such deleterious biological endpoints do not occur.

We have shown, in yeast, that when specific DNA repair pathways are corrupted, a particular level of spontaneous DNA damage accumulates. The resulting genetic instability and loss of biological integrity observed in our strains are analogous to cellular phenotypes of human disease states such as cancer.

For example, cancer cells have been shown to accumulate oxidative DNA damage and display elevated levels of intracellular ROS, polyploidy, genetic instability, aberrant cellular morphologies, and transcriptional alterations (4, 14, 47). In addition to cancer, cells from aged tissue and neurodegenerative diseases accumulate oxidative DNA damage (14). These biological and genetic effects might also occur in cells, such as those affected by chronic inflammation, where conditions of continuous oxidative stress may saturate one or more functional DNA excision repair pathways. We have generated yeast strains possessing different levels of oxidative stress and DNA damage due to deficiencies in one or two DNA excision repair pathways and have established the levels of Ngl1p-recognized lesions that, together with other types of spontaneous DNA damage, are responsible for provoking deleterious biological consequences. Given the similarity of phenotypes observed in several human disease states and our yeast DNA repair mutants, we propose that such strains might be exploited as an informative model system for gauging the sequential biological consequences of chronic oxidative stress and DNA damage accumulation.

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REFERENCES

1. Friedberg, E., Walker, G., and Siede, W. (1995) DNA Repair and Mutagenesis, pp. 2–56, ASM Press, Washington, D. C.
2. Beckman, R. B., and Ames, B. N. (1997) J. Biol. Chem. 272, 19633–19666
3. Doetsch, P. W., Morey, N. J., Swanson, R. L., and Jinks-Robertson, S. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 29–39
4. Hanahan, D. and Weinberg, R. A. (2000) Cell 100, 57–70
5. Lindahl, T., and Wood, R. D. (1999) Science 286, 1897–1905
6. You, H., Swanson, R. L., Harrington, C., Corbett, A. H., Jinks-Robertson, S., Senturker, S., Wallace, S. S., Beiter, S., Dizdaroglu, M., and Doetsch, P. W. (1999) Biochemistry 38, 11298–11306
7. Memisoglu, A., and Samson, L. (2000) Mutat. Res. 451, 39–51
8. Lin, J. J., and Sancar, A. (1999) Biochemistry 28, 7979–7984
9. Snowden, A., Kow, Y. W., and Van Houten, B. (1999) Biochemistry 20, 7251–7259
10. Reardon, J. T., Beets, T., Kung, H. C., Bolton, P. H., and Sancar, A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9463–9468
11. Swanson, R. L., Morey, N. J., Doetsch, P. W., and Jinks-Robertson, S. (1999) Mol. Cell. Biol. 19, 2929–2935
12. Torres-Ramos, C. A., Johnson, R. E., Prakash, L., and Prakash, S. (2000) Mol. Cell. Biol. 20, 3522–3528
13. Gellon, L., Barbee, R., Auffret van den Kemp, P., Thomas, D., and Boeute, S. (2004) Mol. Genet. Genomics 269, 1087–1096
14. Cooke, M. S., Evans, M. D., Dizdaroglu, M., and Lune, J. (2003) FASEB J. 17, 1195–1214
15. Zhang, H., Taylor, J., and Siede, W. (2003) J. Biol. Chem. 278, 9382–9387
16. Poiani, M., Marin, F., Gamba, D., Lucchini, G., and Plevani, P. (1994) Mol. Cell. Biol. 14, 923–933
17. Boehr, V. A., Smith, C. A., Okumoto, D. S., and Hanawalt, P. C. (1995) Cell 80, 359–369
18. Meadows, K. L., Song, B., and Doetsch, P. W. (2003) Nucleic Acids Res. 31, 5560–5567
19. Sambrook, J., and Russell, D. (2001) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
20. Boehr, V. A., and Okumoto, D. S. (1988) in DNA Repair: A Laboratory Manual of Research Procedures (Friedberg, E. C., and Hanawalt, P. C., eds) pp. 347–366, Marcel Dekker, New York
21. Grubs, F. E. (1969) Techonometria 11, 1–21
22. Guo, J., Li, T. K., Koo, D., Nur, E. K. A., and Liu, L. F. (2003) J. Biol. Chem. 278, 15136–15144
23. O’Flourke, T. W., Doudianac, N. A., Makereth, M. D., Doetsch, P. W., and Shadel, G. S. (2002) Mol. Cell. Biol. 22, 4086–4093
24. Aguainoia, H., Gustafsson, L., Rigoulet, M., and Nystrom, T. J. (2001) J. Cell. Chem. 278, 3539–3540
25. Sanchez, Y., Desany, B. A., Jones, W. J., Liu, Q., Wang, B., and Elledge, S. J. (1996) Science 271, 357–360
26. Nyberg, K. A., Michelson, R. J., Putnam, C. W., and Weinert, T. A. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 10200–10205
27. Jorgensen, J. M. (2001) PNAS Microbiol. Rev. 25, 107–123
28. Jiang, Y. W., and Kang, C. M. (2003) Mol. Biol. Cell 14, 5116–5124
29. Chial, H. J., Giddings, T. H., Jr., Siewert, E. A., Hoyt, M. A., and Winey, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10200–10205
30. Jorgensen, J. M., Wang, B., and Zhang, S. Q. (1995) Anal. Biochem. 227, 32–39
31. Tishkoff, D. X., Filosi, N., Gaida, G., and Kolodner, R. (1997) Cell 88, 253–263
32. Greene, C. N., and Jinks-Robertson, S. (1997) Mol. Cell. Biol. 17, 2844–2850
33. Jinks-Robertson, S., and Petes, T. D. (1993) Methods Enzymol. 224, 631–646
34. Finkel, T. (1998) Curr. Opin. Cell Biol. 10, 248–253
35. Finkel, T., and Holbrook, N. J. (2000) Nature 408, 239–247
36. Matunis, M. J. (2002) *Mol. Cell* **10**, 441–442
37. Gasch, A. P., Gartner, L. A., Botstein, D., Brown, P. O., and Eide, D. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 7957–7962
38. Jelinsky, S. A., Estep, P., Church, G. M., and Samson, L. D. (2000) *Mol. Cell. Biol.* **20**, 8157–8167
39. Causton, H. C., Ren, B., Koh, S. S., Harbison, C. T., Kanin, E., Jennings, E. G., Lee, T. I., True, H. L., Lander, E. S., and Young, R. A. (2001) *Mol. Biol. Cell* **12**, 323–337
40. Gilbert, C. S., Green, C. M., and Lowndes, N. F. (2001) *Mol. Cell* **8**, 129–136
41. d’Adda di Fagagna, F., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N. P., and Jackson, S. P. (2003) *Nature* **426**, 194–198
42. Pellizzeri, A., Lee, S. E., Luera, C., Faiani, M., and Haber, J. E. (2001) *Mol. Cell* **7**, 293–300
43. Elledge, S. J., Zhou, Z., Allen, J. B., and Navas, T. A. (1993) *Bioessays* **15**, 333–339
44. Siede, W., Allen, J. B., Elledge, S. J., and Friedberg, E. C. (1996) *J. Bacteriol.* **178**, 5841–5843
45. Basrai, M. A., Velculescu, V. E., Kinzler, K. W., and Hieter, P. (1999) *Mol. Cell. Biol.* **19**, 7041–7049
46. Lew, D. J., and Reed, S. I. (1993) *J. Cell Biol.* **120**, 1305–1329
47. Szatrowski, T. P., and Nathan, C. F. (1991) *Cancer Res.* **51**, 794–798