Ntg2p, a *Saccharomyces cerevisiae* DNA N-Glycosylase/Apurinic or Apurimicidin Lyase Involved in Base Excision Repair of Oxidative DNA Damage, Interacts with the DNA Mismatch Repair Protein Mlh1p

**IDENTIFICATION OF A Mlh1p BINDING MOTIF**

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Ntg2p is a DNA N-glycosylase/apurinic or apurimicidin lyase involved in base excision repair of oxidatively damaged DNA in *Saccharomyces cerevisiae*. Using a yeast two-hybrid screen and a GST *in vitro* transcription and translation assay, the mismatch repair (MMR) protein Mlh1p was demonstrated to interact physically with Ntg2p. The Mlh1p binding site maps to amino acids residues 15–40 of Ntg2p. The Ntg2p binding site is localized in the C-terminal end (483–769) of Mlh1p. Overproduction of Ntg2p results in a mutator phenotype with enhanced frameshift reversion frequency, suggesting partial inhibition of the MMR pathway. In contrast, inactivation of *NTG2* does not enhance mutagenesis, indicating that Ntg2p is not required for MMR. Site-directed mutagenesis of the Mlh1p binding domain of Ntg2p revealed three amino acids (Ser24, Tyr26, Phe27) that are absolutely required for Ntg2p-Mlh1p interaction. These residues are part of a motif found in Ntg2p (Arg23-Ser24, Lys25-Tyr26-Phe27), Exo1p (Arg444-Ser445-Lys446-Phe447), and Sgs1p (Lys583-Ser584-Lys585-Phe586-Phe587). In these three proteins, the motif is part of the domain that interacts with the C-terminal end of Mlh1p. Furthermore, S445A, F447A, and F448A mutants of Exo1p do not bind Mlh1p, but the wild type Exo1p does. Therefore, we propose that the R/K-S/R-Y/F-Y/F sequence could define a Mhl1 binding motif. The results also suggest that base excision repair and MMR can cooperate to prevent deleterious effects of oxidative DNA damage.

Oxidative DNA damage has been involved in pathological processes such as cancer, neurodegenerative diseases, and aging (1–3). It results from the attack of base and sugar moieties by reactive oxygen species generated by cellular metabolism or exogenous agents. Reactive oxygen species can induce several types of lesions in DNA, such as oxidized bases, apurinic/apurimicidin (AP) sites and strand breakage (4, 5). To prevent the deleterious action of oxidative DNA damage, organisms have developed robust DNA repair mechanisms. Reactive oxygen species-induced lesions in DNA bases are primarily repaired by the base excision repair (BER) pathway. BER is a ubiquitous DNA repair process that is initiated by a DNA N-glycosylase removing the altered or unusual bases. Two classes of DNA N-glycosylases are present in the cell: the monofunctional enzymes catalyzing the removal of the lesion, leaving an AP site in DNA; and the DNA N-glycosylases/AP lyases catalyzing both cleavage of the N-glycosyl bond and of the phosphodiester bond to the newly formed AP site (6, 7). The next step in the course of BER is catalyzed by an AP endonuclease generating a single strand break with a 3′-OH end that can be used as a substrate by a DNA polymerase. Finally, BER is completed by successive action of a DNA polymerase and a DNA ligase (8, 9).

In *Saccharomyces cerevisiae*, three DNA N-glycosylases/AP lyases are involved in the repair of oxidatively damaged DNA bases: Ntg1p, Ntg2p, and Ogg1p (10). Ntg1p and Ntg2p are closely related to each other and to *Escherichia coli* Nth (11–15). However, Ntg2p, but not Ntg1p, possesses the consensus sequence for an iron-sulfur center found in most of the Nth homologs (11–15). Cellular localization analysis indicates that Ntg2p is exclusively nuclear, whereas Ntg1p is both nuclear and mitochondrial (14, 16). Furthermore, Ntg1p is DNA damage-inducible, whereas Ntg2p is constitutively expressed (11, 12, 14). Ogg1p does not show significant sequence homology with Ntg1p and Ntg2p except for the helix-hairpin-helix-GPD/K active site domain (17–19). Ntg1p and Ntg2p display a broad substrate specificity, releasing oxidized pyrimidines (11–16) but also purine-derived lesions (13). Although similar, Ntg1p and Ntg2p substrate specificities are not identical (13). Ogg1p exhibits a narrower substrate specificity, catalyzing the removal of two oxidized purines (20). Finally, Ntg1p, Ntg2p, and Ogg1p incise DNA at AP sites via a β-elimination reaction (14, 20). The biological functions of Ntg1p, Ntg2p, and Ogg1p DNA N-glycosylases in *S. cerevisiae* have been investigated by analyzing the phenotypes of mutant strains. Recently, it has

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*This work was supported in part by the Commissariat à l’Energie Atomique and the CNRS. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. § Supported by a fellowship from the Association pour la Recherche sur le Cancer. ¶ To whom correspondence should be addressed: Laboratoire de Radiobiologie du DNA, UMR217 CNRS/CEA, 60–68 avenue du General Leclerc, Fontenay-aux-Roses 92265, France. Tel.: 33-1-4654-8858; Fax: 33-1-4654-8859; E-mail: boiteux@dsvidf.ceaf.fr.

1 The abbreviations used are: AP, apurinic/apurimicidin; AD, activation domain; BER, base excision repair; Can, canavanine resistance; DDB, DNA binding domain; DHT, 5,6-dihydrothymidine; GST, glutathione S-transferase; IVTT, *in vitro* transcription-translation; Me-FapyG, 2,6-diamino-4-hydroxy-5-N-methylformamidopurine; MMR, mismatch repair; NER, nucleotide excision repair; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
been reported that an ntg1 ntg2 double mutant strain is not unusually sensitive to H$_2$O$_2$, nor does it exhibit a mutator phenotype (15, 21). However, a ntg1 ntg2 rad14 triple mutant exhibits a mutator phenotype and enhanced sensitivity to the lethal effect of chemical oxidants compared with that of a wild type strain (21). These results show that oxidatively damaged pyrimidines are not only removed by BER enzymes such as Ntg1p and Ntg2p but also by the nucleotide excision repair (NER) pathway. It was demonstrated recently that the Apn1p can also incise DNA on the 5’-side of various oxidatively damaged bases (22). On the other hand, inactivation of the Ogg1p results in a spontaneous GC to TA mutator phenotype, which is probably caused by the accumulation of 8-oxoG in DNA (23, 24).

In S. cerevisiae, recent studies strongly suggest that the mutagenic action of endogenous oxidative DNA damage is also counteracted by the mismatch repair pathway (MMR) (25–28). A genetic analysis showed that reversion rates for three classes of base substitutions are greatly enhanced in yeast strains deficient in Msh2p or Msh6p; however, when cells are grown anaerobically, the reversion rates for these base substitutions, in those strains, are strongly reduced (25). Moreover, Ogg1p msh2 and ogg1 msh6 double mutants exhibit a synergistic increase in GC to TA spontaneous transversions compared with the ogg1, msh2, and msh6 single mutants (26). These last results suggest that MMR eliminates adenine residues incorporated opposite 8-oxoG (26). MMR could also remove 8-oxoG incorporated by a DNA polymerase using 8-oxo-dGTP as a precursor in the course of DNA replication. Therefore, MMR could be the functional homolog in S. cerevisiae of the bacterial MutY and MutT (26, 29).

Table I

| Strain                  | Genotype                   | Source/reference |
|-------------------------|----------------------------|------------------|
| RKY3109                 | MATa ura3–52, leu2Δ1, trp1Δ63, his3Δ200, lys2Δ8gl, hem3–10, ade2Δ1, ade8 | P. Bertrand      |
| BG301                   | RKY3109 with mhl1Δ–kanMX6  | This work        |
| BG307                   | RKY3109 with exo1Δ–kanMX6  | This work        |
| BG309                   | RKY3109 with nlg2Δ–kanMX6  | This work        |
| BG312                   | RKY3109 with mhl1Δ–kanMX6, ntg2Δ–TRP1 | This work        |
| BG308                   | Y190 with pms1Δ–kanMX6     | This work        |
| BG313                   | Y190 with msh2Δ–kanMX6     | This work        |

Materials and Methods

Bacterial and Yeast Strains—Microbiological techniques and media used in this study have been described by Miller (30) and Sherman (31) for E. coli and S. cerevisiae, respectively. Bacterial strains DH5α and JM105 were used for plasmid construction and preparation (32). Yeast two-hybrid tests were performed in strain Y190 (MATa gal4 gal80 his3 Δ trp1–901 ade2–101 ura3–52 leu2–3, 112 URA3::GAL1::lacZ LYS2::GAL4::UAS::HIS3 can5) (33). Other S. cerevisiae strains used in this study are listed in Table I. Deletion mutants of MLH1, PMS1, MSH2, NTG2, and EXO1 were constructed by the PCR-mediated one-step replacement technique using the kanMX module (34, 35). Yeast transformations were performed using the polyethylene glycol/lithium method (36). Gene replacement was confirmed by PCR analysis on genomic DNA.

Phusion Constructs—Full-length and truncated NTG1, NTG2, MLH1, and EXO1 open reading frames were prepared by PCR amplification from yeast genomic DNA using the Phusion DNA polymerase (Stratagene) and specific primers (Genosys) harboring the BamHI and SmaI restriction sites allowing in-frame insertion of the open reading frames into the vectors. Yeast two-hybrid plasmid vectors are pGBT9 (37) and pAS2ΔΔ (38) yielding bait constructs and pACT2 for prey constructs. Both constructs express Gal4p (NTG2, Activation Domain) C-terminal fusion proteins. Prey constructs express Gal4pAD (Activation Domain) C-terminal fusion proteins. Plasmids pGEX4T-1 (Amersham Biosciences) and pBluescript II KS (Stratagene) were used for GST-in vitro transcription-translation (IVTT) assays. Plasmid PTC99nA (Amersham Biosciences) was used to express and purify full-length Ntg2p and truncated Ntg2p(31–380). Finally, pYX121 (R&D) was used to express native Ntg2p. All constructs were confirmed by sequencing. Sequences of plasmids, construction schemes, and oligonucleotide primers are available upon request.

Site-directed Mutagenesis—Point mutations were performed by PCR-mediated mutagenesis (QuickChange site-directed mutagenesis Kit, Stratagene). Alanine substitutions at the N-terminal end of Ntg2p are designated pASΔΔ–NTG2 as target and amino-acids. The plasmid expressing Gal4pΔNTG2(1–20) was constructed by changing Gin297Val298Arg299 of Ntg2p into three stop codons. The plasmid expressing Gal4pNDNtg2p(1–731) was constructed by changing Glu732 His733Val734 into stop codons. All constructs were confirmed by sequencing. Sequences of plasmids, construction schemes, and oligonucleotides primers are available upon request.

Yeast Two-hybrid Screening and β-Galactosidase Assays—Yeast two-hybrid screening was performed with the FYR1 genomic library (38) containing randomly sheared genomic DNA fragments of 700 bp mean size in a modified pACT2 vector. The library DNA was purified by ultracentrifugation in a CsCl gradient to obtain strain Y190 containing the Gal4pΔNtgp2p bait fusion. Transformed cells were plated directly onto 100 μg/ml SD adenine minimal medium plates containing 25 or 50 μg/ml 3-amaminotriazole to select His+ strains. Colonies growing after 4–7 days were streaked on SD + adenosine + histidine and assayed for β-galactosidase production in an overlay plate assay. Blue colonies were restreaked on the same medium and tested again for β-galactosidase production. Among more than 1.1 × 107 transformants tested for histidine prototrophy and positive β-galactosidase assays, positive clones were selected. Prey plasmid DNA was recovered and transformed in E. coli strain 1086 (trpC–9830 leuB6 pyrF74:Trn5 ΔlacI 1 POZYA–74 galU galK hisD20 rpsL) selecting the yeast LEU2 marker. Alternatively, we prepared total yeast DNA by using a QIAamp kit (Qiagen, Chatsworth, CA), amplified the prey DNA by PCR, and purified the product on a QIAquick column. After sequencing, the identity of the insert was determined by using the S. cerevisiae Genome Data base (http://genome-www2.stanford.edu/cgi-bin/hgi-blast). β-Galactosidase liquid assays were performed by resuspending cells in 1 ml of Z buffer with 0.8 μg of o-nitrophenyl β-D-galactosidase substrate plus chloroform and SDS. Reaction were stopped with Na2CO3 when an appropriate level of color had developed, and β-galactosidase activity was measured (30). The average and S.D. of at least three independent experiments are presented.

GST-IVTT Assay—GST and GST-Ntg2p were expressed in E. coli JM105 harboring a pGEX4T-1 or pGEX-NTG2, respectively. Cells were grown at 37 °C in LB broth medium (1 liter) containing 100 μg/ml ampicillin, until the A$_{600}$ = 0.7 and induced for 16 h at 20 °C in the presence of 0.1 μL isopropyl-1-thio-β-D-galactosidase. Cells were collected and stored at –80 °C. Cell pellets were resuspended into 10 mL lysis buffer (20 mM Tris-HCl, pH 8.2, 1 mM NaEDTA, 500 mM NaCl, 0.8 μg/ml antipain, 0.8 μg/ml leupeptin, 0.8 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Cell suspensions were sonicated and centrifuged at 4 °C. The supernatant fractions were dialyzed against phosphate-buffered saline buffer and applied to glutathione-Sepharose 4B (Amersham Biosciences) equilibrated with phosphate-buffered saline buffer for 1 h at room temperature under gentle mixing. The GST-methionine-labeled Mlh1p was added to equivalent amounts of GST or GST-Ntg2p bound to the glutathione-Sepharose 4B beads in 200 μl of buffer (50 mM Tris-HCl, pH 7.5, 0.1% Nonidet P-40, 150 mM NaCl, 1 mM NaEDTA, 0.3 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). The mix-
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Ntg2p Interacts with Mlh1p in the Yeast Two-hybrid Assay—Using full-length Ntg2p (Fig. 1A) as bait in a two-hybrid screen of 1.1 × 10^6 transformants, 15 positive colonies were selected and analyzed by sequencing. The three strongest interactions, based on the early appearance of colonies on (His^+ + 3-amino-triazole) plates and on the intensity of color in the X-gal overlay plate assay, were found to match two fragments of the C-terminal end (402–769) and (483–769), from the MMR protein, Mlh1p (Fig. 1, B and C). Interestingly, the C-terminal end of Mlh1p also interacts with Pms1p, Mlh2p, Mlh3p, Exo1p, or Sgs1p in two-hybrid assays (44–46). Because the Mlh1p prey were truncated, it was important to test the ability of full-length Mlh1p to interact with Ntg2p. Fig. 1, B and C, shows that the full-length Mlh1p interacts with Ntg2p in a two-hybrid assay as well as the truncated Mlh1p (483–769) does. Because Ntg2p and Ntg1p present a high degree of similarity, we also performed a two-hybrid assay with full-length Ntg1p as bait and full-length Mlh1p as prey. Fig. 1B shows that Ntg1p does not interact with Mlh1p by two-hybrid assay. We also constructed a Mlh1p (1–731) mutant with its 38 last amino acids deleted and tested it for interaction with the full-length Ntg2p by the two-hybrid system. Fig. 1C shows that the Mlh1p (1–731) does not interact with Ntg2p. Control experiments show that neither Ntg2p bait nor full-length or truncated Mlh1p prey alone stimulate reporter gene expression to allow detectable growth on medium lacking histidine or significant X-gal coloration (Fig. 1, B and C, and data not shown). Therefore, these results demonstrate an interaction between Ntg2p and Mlh1p and reveal a physical link between BER and MMR in S. cerevisiae (Fig. 1D).

Ntg2p Physically Interacts with Mlh1p in Vitro—To demonstrate a direct interaction between Ntg2p and Mlh1p, we used the GST-IPTG bait prey binding assay. [35S]Methionine-labeled Mlh1p was expressed from an IPTG vector (Fig. 2, lane 1). GST or GST-Ntg2p was immobilized on glutathione-Sepharose beads and incubated in the presence of the IVTT [35S]methionine-labeled Mlh1p to allow binding. Afterward, beads were washed, and the GST-pull down products were separated on SDS-PAGE. Fig. 2 shows that [35S]Methionine-labeled Mlh1p was retained specifically on the GST-Ntg2p beads (lane 3), whereas it did not bind on the GST beads (lane 2). Therefore, the results obtained with the GST-IPTG methodology confirm the yeast two-hybrid results presented above and demonstrate that full-length Ntg2p can interact directly with full-length Mlh1p.

The N-terminal Region of Ntg2p Interacts with the C-terminal Region of Mlh1p—Initial screening of the yeast interaction trap library allowed us to show that full-length Ntg2p interacts with the C-terminal end (483–769) of Mlh1p (Fig. 1D). To determine the region of Ntg2p required for the interaction with Mlh1p, a series of deletions was constructed at the N- or C-terminal ends of Ntg2p (Fig. 3). The various Gal4pAD–Ntg2p deletion mutants were used as bait in a two-hybrid assay with full-length Gal4pAD–Mlh1p (1–769) or truncated Gal4pAD–Mlh1p (483–769). Western blot analysis shows that all Ntg2p deletion mutants are expressed at a level similar or higher than that of the full-length Ntg2p (Fig. 3A). A yeast interaction trapping assay shows that deletion of the first 31 amino acids at the N-terminal end of Ntg2p abolishes the interaction with Mlh1p, either full-length or truncated (Fig. 3B). On the other hand, large deletions at the C terminus of Ntg2p do not alter the interaction between Ntg2p and Mlh1p. Indeed, the first 40 amino acids of Ntg2p (1–40) are sufficient for interaction with Mlh1p (Fig. 3B). Therefore, these results define residues 15–40 as the minimal region of Ntg2p required to interact with Mlh1p (Fig. 3B).

Overexpression of Ntg2p Reduces in Vivo DNA MMR—The MutLo heterodimer, composed of Mlh1p and Pms1p, is required for a functional MMR in S. cerevisiae (47–50). Pms1p binds the C-terminal region of Mlh1p (501–769), which also contains the Ntg2p binding domain (44). Other partners of Mlh1p, such as Mlh2p, Mlh3p, Exo1p, and Sgs1p, also bind its C terminus (45, 46, 51). Therefore, blocking the C terminus of Mlh1p by overexpression of Ntg2p could interfere with the MMR function in the cell. To test this hypothesis, we have measured spontaneous forward mutation to Can^R and reversion at the hom3–10 and lys2-Bgl markers in yeast cells that overexpress Ntg2p. The hom3–10 and lys2-Bgl alleles allow measurement of –1 and –4 frameshifts, respectively, and serve...
**FIG. 1.** *Ntg2p Interacts with Mlh1p in S. cerevisiae*

**A.** Schematic representation of the yeast Ntg2 protein. The active site and the DBDs are represented. The full-length Ntg2p was used for two-hybrid screening.

**B.** Specific interaction of Ntg2p with Mlh1p. Yeast Y190 cells hosting various constructs of pGBT9 and pATC2 vectors were grown at 30 °C on synthetic media lacking tryptophan, leucine, and histidine and

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*Ntg2p Interacts with Mlh1p in a yeast two-hybrid assay. A, schematic representation of the yeast Ntg2 protein. The active site and the DBDs are represented. The full-length Ntg2p was used for two-hybrid screening. B, specific interaction of Ntg2p with Mlh1p. Yeast Y190 cells hosting various constructs of pGBT9 and pATC2 vectors were grown at 30 °C on synthetic media lacking tryptophan, leucine, and histidine and*
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Ntg2p Is Not Required for DNA MMR—The interaction between Ntg2p and Mlh1p could suggest that these two repair proteins act in the same repair pathway. The requirement of Ntg2p for MMR was not expected because Ntg2p-deficient strains do not present a detectable spontaneous mutator phenotype (15, 21). However, the impact of the Ntg2p on frameshift mutations, which are the hallmark of MMR deficiencies, had not been investigated. Table III shows that deletion of the NTG2 gene does not affect Hom− and Lys+ reversion frequencies, which remain similar to those of a wild type strain (Table III). Furthermore, spontaneous mutant frequencies in a ntg2 mlh1 double mutant are similar to those of a mlh1 single mutant (Table III).

The Mlh1p Interacting Domain of the Ntg2p Is Not Required for Its DNA N-Glycosylase Activity in Vitro—Sequence analysis suggests that the N-terminal end of Ntg2p is not involved in catalysis or in DNA binding (Fig. 1A). However, it was interesting to measure the enzymatic activity of Ntg2p mutants unable to bind Mlh1p such as Ntg2p(31–380). The various constructs were expressed in a wild type strain of S. cerevisiae, and DNA N-glycosylase/AP lyase activity was measured in crude protein extracts using a 34-mer DNA duplex containing a single 5,6-dihydrothymidine (DHT-A), as substrate. Fig. 4A shows that in our assay conditions, there is cleavage of about 15% of the DHT-containing strand by extracts of yeast cells hosting the empty vector. This activity is presumably caused by endogenous Ntg2p and Mlh1p. On the other hand, a significant increase in DHT-containing strand cleavage activity is observed in extracts of yeast cells hosting vectors that express the wild type Ntg2p(1–380) or the mutant Ntg2p(31–380) (Fig. 4A). In addition, using the purified proteins in a Me-FapyD DNA N-glycosylase assay, we show that Ntg2p(31–380) exhibits an activity identical to that of a full-length Ntg2p (Fig. 4B). Thus, loss of the Ntg2p-Mlh1p interaction domain does not prevent Ntg2p from maintaining a repair function in vitro.

Identification of Ntg2p Amino Acids Required for Interaction with Mlh1p—From the Ntg2p deletion mapping, N-terminal amino acids (15–40) were suggested to be sufficient to maintain the two-hybrid interaction with Mlh1p (Fig. 3B). In an effort to localize the Ntg2p amino acids important for Ntg2p-Mlh1p interaction, a series of site-directed substitution mutants was generated from the pAS2Δ-NTG2 construct. Mutant Gal4pDBD-NTG2p(1–380) forms were tested for interaction with full-length Mlh1p using a yeast two-hybrid assay and a Hom− reversion assay. The initial strategy used was based on modification of the charged amino acids because they represent more than 50% of the Ntg2p residues between positions 15 and 40 (Fig. 5A). Surprisingly, neither the D16A,E18A,E19A,E21A nor the R23A,K25A,K28A,K29A substitutions completely abolished the interaction with Mlh1p (Fig. 5B). However, R23A and K25A Ntg2p mutants present a significant decrease in β-galactosidase activity and Hom− reversion frequency compared with the wild type (Fig. 5B). Although not required for the Ntg2p-Mlh1p interaction, these residues are probably involved in the binding domain. Because modification of charged amino acids of Ntg2p did not abolish Mlh1p binding, we decided to modify the Tyr20 and Phe27 aromatic amino acids. Interestingly, such residues are not present in the N terminus of the Ntg1p (Fig. 5A). In fact, the N-terminal ends of Ntg1p and Ntg2p present no homology, whereas Ntg1p and Ntg2p present an overall

supplemented with 25 mM 3-aminotriazole. Cells in each section were streaked from a single transformant that was colony-purified. Labels indicate the fusion proteins present in the Y190 cells in the order: Gal4p DBD fusion (bait)/Gal4p AD fusion (prey). (−) indicates the absence of bait or prey. Ntg2p, Ntg1p, and Mlh1p were full-length proteins. C, interaction between the full-length Ntg2p (bait) with full-length or truncated Mlh1p (prey). Patches of one transformant containing each indicated pair of baits and prey were tested for β-galactosidase production in an overlay plate assay. (−) indicates control in absence of prey. D, schematic representation of the yeast Mlh1 protein with the Ntg2p binding domain obtained from two-hybrid experiments.

as diagnostic for defects in MMR (52). In contrast, forward mutation at CAN1 reflects a wide variety of inactivating mutations. Expression of the native Ntg2p from the pTX212-NTG2 construct, where NTG2 is placed under the control of the strong TPI promoter, results in enhanced frequencies of CanR mutants, Hom− and Lys+ revertants (Table II). Furthermore, the presence of an excess of Gal4pDBD-Ntg2p also results in a mutator phenotype that is dependent upon the NTG2 expression level because cells hosting pAS2Δ-NTG2 (strong full-length ADH1 promoter) show a 35-fold increase in Hom− revertants, whereas those hosting pGBT9-NTG2 (weak truncated ADH1 promoter) only show a 9-fold increase in Hom− revertants (Table II). These results show that high levels of Ntg2p can inhibit a cellular process involved in genetic stability. Although significant, the mutator phenotype obtained with the highest Ntg2p expression does not reach the level observed for a strain in which the MLH1 gene has been deleted (Table II).

To investigate whether the Ntg2p-mediated mutator effect occurs via the Ntg2p-Mlh1p interaction, the N- and C-terminal deletion mutants of Ntg2p were overexpressed, and the mutation frequencies were measured. The results show that all of the Ntg2p forms able to interact with Mlh1p (Fig. 3B) induce a mutator phenotype (Table II). In contrast, expression of Ntg2p(1–20) or Ntg2p(31–380), which do not interact with Mlh1p, do not enhance mutant frequencies (Table II). It should be noted that the mutator phenotypes obtained from two positive interaction deletion mutants, Ntg2p(1–40) and Ntg2p(1–70), were intermediate (Table II). Finally, overproduction of Ntg2p in an mlh1 background does not affect mutant frequencies (Table II; mlh1Δ entries). Taken together, these data reveal a strict correlation between the capacity of Ntg2p to interact with Mlh1p and the induction of a spontaneous mutator phenotype. Therefore, they strongly support the idea of a competition for the C-terminal region of Mlh1p between Ntg2p and other partner(s).

IVTT

| GST-Ntg2 | MLH1 |
|----------|------|
| GST      | +    |

Lac1 2 3

Fig. 2. Mlh1p binds to Ntg2p in vitro. Fusion products of Ntg2p (GST-Ntg2p) were bound to glutathione-Sepharose 4B beads and incubated with 35S-labeled full-length Mlh1p generated by IVTT (see “Materials and Methods”). As a control for nonspecific binding to the beads, the IVTT 35S-labeled Mlh1p was incubated with the GST protein. After washing, the proteins were eluted from the beads, separated by SDS-PAGE, and Mlh1p was visualized by autoradiography.
similarity of 60% (10, 14). The results show that Y26A and F27A mutants of Ntg2p have no detectable β-galactosidase activity in the two-hybrid assay with Mlh1p (Fig. 5B). Furthermore, the overexpression of the Y26A or F27A mutant form of Ntg2p does not result in an enhanced Hom reversion frequency (Fig. 5B). To complete our study, we also generated an S24A mutant of Ntg2p and analyzed its properties. Fig. 5B shows that the S24A mutation abolishes the interaction between Ntg2p and Mlh1p. The expression of all of the point mutants used in this study was confirmed by Western blotting analysis using anti-Gal4pDBD antibodies (data not shown and Fig. 6B). These results led us to conclude that Ser24, Tyr26, and Phe27 of Ntg2p are required for the interaction with Mlh1p.

Identification of a Ntg2p-like Mlh1 Binding Motif in Exo1p—The results reported in this study showed that Ser24, Tyr26, and Phe27 of Ntg2p are essential for the interaction with Mlh1p (Fig. 5). They also suggest that Arg23 and Lys25 of Ntg2p are involved in the Mlh1p interacting domain. We hypothesized that the sequence R/K-S-R/K-Y/F-Y/F, composed of residues 23–27 of Ntg2p, could constitute a Mlh1p binding motif. Searches in the S. cerevisiae sequence data base revealed that Exo1p and Sgs1p, two Mlh1p partners, possess such a motif (Fig. 6A). Importantly, those residues in both Exo1p (R-K-F-F) (444–448) and Sgs1p (K-S-K-F-S) (1382–1386) are located in the Mlh1p binding domain (46, 51). Furthermore, Exo1p and Sgs1p, as well as Ntg2p, interact with the C-terminal end of...
Therefore, amino acids Ser445, Phe447, and Phe448 of Exo1p are involved in the Exo1p-Mlh1p interaction, supporting the concept of a Mlh1p binding motif.

To test the hypothesis of a consensus motif for Mlh1p binding, we generated a construct that expresses the C-terminal end of Exo1p(400–702) fused to the Gal4p DBD. Using the yeast two-hybrid assay, we confirmed that the Gal4pDBD-Ntg2p(1–20) 19.1 (1.5) 0.25 (0.7) ND

The production of the various mutant forms of Ntg2p or Exo1p was demonstrated by Western blotting analysis (Fig. 6B). Moreover, overexpression of the C-terminal end of Exo1p generates a Hom− mutator phenotype, whereas expression of the Exo1p(F447A) mutant does not (data not shown). Therefore, amino acids Ser445, Phe447, and Phe448 of Exo1p are involved in the Exo1p-Mlh1p interaction, supporting the concept of a Mlh1p binding motif.

DISCUSSION

In S. cerevisiae, cooperation for the removal of DNA damage between DNA repair pathways such as BER, NER, and MMR, has been demonstrated (53). Oxidative DNA base damage is primarily repaired by the BER pathway initiated by DNA N-glycosylases such as Ntg1p, Ntg2p, and Ogg1p. However, Ntg2p was shown to act synergistically with Ntg1p and Ntg2p-mediated BER, to release oxidatively damaged pyrimidines (21). Furthermore, Ogg1p and MMR are both involved in the prevention of the mutagenic consequences of 8-oxoG residues in DNA (26, 29). The various repair pathways can act independently, and the synergism could result from their overlap.

![Fig. 4. DNA N-glycosylase assays. A, cleavage of a DNA duplex containing a single 5,6-dihydrothymidine (DHT-A) by cell-free protein extracts. A 34-mer DNA duplex containing a single DHT was used as a substrate. The DHT-containing strand was 32P-labeled at the 5′-end before annealing with a complementary sequence yielding the DHT-A duplex. The substrate was incubated for 30 min at 37 °C with cell-free protein extracts (3.5 μg) from yeast Y190 hosting pAS2Δ control vector, or plasmids that overproduce the wild type Ntg2p(1–380) or the deletion mutant Ntg2p(31–380). The products of the reaction were separated by 20% PAGE in the presence of 7 M urea, and cleavage was quantitated by phosphorimaging. B, Me-FapyG DNA N-glycosylase activity assay on purified proteins. Ntg2p or Ntg2p(31–380) protein was assayed using [3H]FapyG-poly(dG-dC) as substrate. Various amounts of enzymes were incubated with the substrate for 15 min at 37 °C.](image-url)
ping substrate specificities for DNA damage. Alternatively, the repair systems can cooperate for the removal of DNA damage in a way that requires physical interactions between protein components. In *S. cerevisiae*, Msh2p, a key protein in MMR, was showed to interact physically with Rad14p, an essential NER component (54). Furthermore, the human 3-methyladenine DNA N-glycosylase interacts physically with hHR23A and hHR23B, which play a critical role in NER in human cells (55). Finally, another study showed that hMYH, a DNA N-glycosylase involved in BER, physically and functionally interacts with the MMR Msh2p-Msh6p heterodimer via the hMsh6p subunit in human cells (56).

In this study, we report a physical interaction between Ntg2p, a DNA N-glycosylase/AP lyase involved in oxidative DNA base damage, and Mlh1p, an essential component of the MMR pathway. Physical interaction between Ntg2p and Mlh1p is demonstrated by yeast two-hybrid screening and *in vitro* GST pull-down experiments. Direct interaction is consistent with the fact that Ntg2p-Mlh1p interaction is maintained in a Δmsh2 or a Δpms1 context (data not shown). The Ntg2p-Mlh1p interaction is also specific because neither Ntg1p nor Ogg1p was found to interact with Mlh1p (data not shown). We mapped the Ntg2p-Mlh1p interacting domains using a series of deletion mutants. In Ntg2p, the domain sufficient to maintain the two-hybrid interaction with Mlh1p is mapped at the N-terminal end, residues 15–40. This localization can explain why Ntg2p binds Mlh1p and Ntg1p does not. Indeed, the N-terminal ends of Ntg2p and Ntg1p are completely different, whereas other regions present very high similarities. On the other hand, the C-terminal end, residues 483–769, of Mlh1p is sufficient to interact with Ntg2p. It should be noted that the C-terminal region of Mlh1p, which contains the Ntg2p binding domain, is also required for the binding of other partners such as Pms1p, Mlh2p, Mlh3p, Exo1p, and Sgs1p (45, 46, 51). These results strongly point to a competition between the various protein partners for the binding of the C-terminal end of Mlh1p in *S. cerevisiae*.

To address the question of the Ntg2p-Mlh1p interaction in the cellular context, we have constructed *ntg2* and *mlh1 ntg2* deletion mutants. Our genetic experiments indicate that Ntg2p does not play a critical role in MMR because neither the *ntg2* strain nor *ntg2 mlh1* strain shows an increase in global or specific MMR mutagenesis, compared with the wild type and *mlh1* strains, respectively. The results also show that deletion of NTG2 does not affect Hom+ reversion events, indicating that the physiological level of Ntg2p does not influence MMR function. However, we cannot exclude the possibility that Ntg2p could participate in MMR but would be redundant with enzymes containing equivalent properties. On the other hand, the interaction between Ntg2p and Mlh1p can be beneficial to BER by coordination of repair proteins by Mlh1p. A recent study reported that the adenine DNA N-glycosylase activity of hMYH

**Fig. 5. Effect of missense mutations in the Mlh1p interaction region of Ntg2p.** A, alignment of the N-terminal amino acid sequences of the *S. cerevisiae* Ntg1p and Ntg2p. Identical amino acids are in black boxes, and similar amino acids are in gray boxes. The amino acids of the Ntg2p which are mutated to alanine are indicated below the alignment by an asterisk. Arrows indicate the Ntg2p amino acids essential for the interaction. B, interaction of Ntg2p mutants with Mlh1p was quantitated by β-galactosidase activities in a two-hybrid assay. Wild type and mutant Gal4pDBD-Ntg2p(1–380) were used as bait, and Gal4pAD*-Mlh1p(1–789) was used as prey. Units are as described under “Materials and Methods,” and the values shown represent averages and S.D. values from three independent experiments. Hom+ revertant frequencies in RKY3109 strain hosting pAS2ΔΔ-NTG2 plasmids were determined as described. Values presented are relative to the mutant frequency observed for the wild type strain containing the vector pAS2ΔΔ only, which is 0.35 × 10⁻⁷. ND, not determined.
can be stimulated by the hMutSα complex, thus demonstrating that protein-protein interactions are important in modulating BER system efficiency (56). The proposed biological function of the hMYH-hMutSα interaction would be to target hMYH on the newly synthesized DNA strand to eliminate adenine incorporated opposite 8-oxoG (56). In human cells, Mlh1p was also shown to interact with Med1p, another protein that, like Ntg2p, belongs to the endo III super family (57). By similarity, we hypothesized that Mlh1p can target Ntg2p to the newly synthesized DNA strand to release oxidatively damaged pyrimidines incorporated by DNA polymerases using damaged precursors such as 5-OH-dCTP (58). Again, the lack of detectable phenotype of Ntg2p-deficient strains does not support this hypothesis. As discussed before, obtaining a clear phenotype might require the simultaneous inactivation of redundant functions. For example, enhanced sensitivity to the killing effect of chemical oxidants requires the simultaneous inactivation of BER enzymes such as Ntg1p, Ntg2p, Apn1p, and of the NER pathway (15, 21, 22).

To investigate the impact of the level of Ntg2p on MMR, we overexpressed the Ntg2p in wild type strain. Our results show that the overproduction of Ntg2p results in a mutator phenotype with enhanced Hom+ revertant frequency, suggesting partial inhibition of the MMR pathway. The mutator phenotype is strictly correlated with the ability of the Ntg2p mutants to bind Mlh1p in yeast two-hybrid assay. We suggest that the overproduced Ntg2p can sequester Mlh1p and prevent the participation of the others partners in MMR. Although significant, the Ntg2p-induced mutator phenotype is much weaker than that expected from the complete inactivation of MMR. This last result may suggest that Ntg2p does not compete with an essential component of MMR such as Pms1p but rather with other partner(s) such as Exo1p. Indeed, deletion of EXO1 results in a relatively modest, about 10-fold, increase in Hom+ revertants (46, 59). These results indicate that the expression of gene such as NTG2 may be strongly regulated to ensure a low mutation rate. This is consistent with the expression level of the Ntg2p, which is constitutively expressed at a relatively low level, contrary to its homolog Ntg1p, which is inducible by oxidizing agents (11, 12, 14).

The finding of multiple Mlh1p partners points to Mlh1p as a “molecular matchmaker,” coordinator, and regulator of the MMR proteins and coupling this pathway to replication machinery (60). In S. cerevisiae, Mlh1p has been demonstrated to interact directly with protein partners such as Pms1p, Mlh2p, Mlh3p, Exo1p, Sgs1p, and Ntg2p. All of them interact with the C-terminal domain of Mlh1p (44–46, 51). Therefore, the multiplicity of Mlh1p partners implies the formation of alternative complexes in the cell. One can distinguish two different groups among Mlh1p partners: Pms1p, Mlh2p, and Mlh3p, which are required for specialized MMR functions; and Exo1p, Sgs1p, and Ntg2p, which are not strictly required for MMR (which are dispensable but may be required for MMR). In this study, dissection of the Ntg2p binding domain led us to propose a putative Mlh1p binding motif with the consensus R/K-S-K-Y/F-F. This motif is found in the Mlh1p-interactive domain of Ntg2p, Exo1p, and Sgs1p, whereas it does not exist in Pms1p, Mlh2p, or Mlh3p. Therefore, we suggest two binding sites at the C-terminal end of Mlh1p. The presence of two sites is also suggested by a recent study showing that Mlh1p-Pms1p-Exo1p can form a heterotrimer (46). The fact that overexpression of Ntg2p has an effect on MMR could also be explained by the
formation of an excess Mlh1p-Pms1p-Ntg2p heterotrimer, and a deficit in the other heterotrimers, such as Mlh1p-Pms1p-Exo1p or Mlh1p-Pms1p-Sgs1. Similarly, the overexpression of the Mlh1p results in the inhibition of MMR in *S. cerevisiae* (61). Taken together, these results strongly suggest that the level of expression of Mlh1p in relation to its partners must be tightly regulated because an imbalance in the cellular amount of one partner can result in alteration of the MMR or other repair pathways.

The Mlh1p binding motif described in this study may be limited to *S. cerevisiae*. Alternatively, it may have a general value in eukaryotes. The fact that hNth1p, the human functional homolog of Ntg2p, does not contain such a motif argues against the generality of these results. However, Med1p, which is also a human DNA N-glycosylase from the Nth1 family, possesses a degenerated Mlh1p binding motif (T-S-L-Y-F) in the region that interacts with hMlh1p (57). In addition, the Mlh1p binding motif described in this study is conserved in the human Exo1p (R-S-R-F-F) and in a degenerated form in the human Blamp (Bloom syndrome, the human homolog of Sgs1p) (S-S-H-Y-F), suggesting a possible conservation of this motif through evolution.

Acknowledgments—We thank the Comité de Radioprotection of Electricité de France. Thanks to Dr. J. P. Radicella and F. Le Page for critical reading of the manuscript. We thank Dr. J. Cadet (CEA-Grenoble, France) for the DHT-containing 34-mer and Dr. P. Bertrand for yeast strains and fruitful discussions. We thank C. Bossieho for technical expertise in the two-hybrid screen.

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