MPH1, A Yeast Gene Encoding a DEAH Protein, Plays a Role in Protection of the Genome From Spontaneous and Chemically Induced Damage

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ABSTRACT

We have characterized the *MPH1* gene from *Saccharomyces cerevisiae*. *mph1* mutants display a spontaneous mutator phenotype. Homologs were found in archaea and in the EST libraries of Drosophila, mouse, and man. Mph1 carries the signature motifs of the DEAH family of helicases. Selected motifs were shown to be necessary for *MPH1* function by introducing missense mutations. Possible indirect effects on translation and splicing were excluded by demonstrating nuclear localization of the protein and splicing proficiency of the mutant. A mutation spectrum did not show any conspicuous deviations from wild type except for an underrepresentation of frameshift mutations. The mutator phenotype was dependent on *REV3* and *RAD6*. The mutant was sensitive to MMS, EMS, 4-NQO, and camptothecin, but not to UV light and X rays. Epistasis analyses were carried out with representative mutants from various repair pathways (*msh6*, *mag1*, *apn1*, *rad14*, *rad52*, *rad6*, *mms2*, and *rev3*). No epistatic interactions were found, either for the spontaneous mutator phenotype or for MMS, EMS, and 4-NQO sensitivity. *mph1* slightly increased the UV sensitivity of *mms2*, *rad6*, and *rad14* mutants, but no effect on X-ray sensitivity was observed. These data suggest that *MPH1* is not part of a hitherto known repair pathway. Possible functions are discussed.

CELLS have evolved a broad arsenal of repair mechanisms to defy threats to the integrity of their genetic information (for overview see Friedberg *et al.* 1995). Chemical modifications of DNA constitutents by environmental and intracellular factors such as radiation or chemical agents may alter the informational content of the DNA or lead to cell death by blocking transcription or replication. A variety of general pathways coping with such lesions have been defined so far. In base excision repair, the damaged base is removed by a specific glycosylase, the resulting apurinic or apyrimidinic site is incised by an AP-lyase activity or an AP-endonuclease, the abasic sugar is removed, and an undamaged nucleotide is inserted using the information of the complementary strand. Nucleotide excision repair, which is mainly involved in the removal of bulky adducts, excises an oligonucleotide containing the damage by cutting several nucleotides to the left and right of the damage on one strand. The oligonucleotide is removed by a helicase and the remaining gap is filled by DNA polymerase. Recombinational repair corrects damage by recombination with a homologous double-stranded DNA molecule. This type of repair is particularly important if both strands of a DNA double helix are damaged in the same region, *e.g.*, by a double-strand break or an interstrand cross-link.

Damage tolerance mechanisms allow the cells to proceed with vital processes such as transcription and replication even in the presence of DNA damage. The price the cells have to pay is an increase in the probability of acquiring a mutation, since these mechanisms involve the mutagenic bypass of lesions that block normal replicative DNA polymerases (Bridges 1999). This does not result in removal of the damage, but allows the cells to continue growth, if repair is not fast enough. In *Escherichia coli*, lesion bypass synthesis is carried out by the UmuD′$_2$C complex (pol V), which is formed in response to DNA damage after proteolysis of UmuD by activated RecA (Tang *et al.* 1999). In the yeast *Saccharomyces cerevisiae*, several enzymes are involved in translesion synthesis. The *REV1* gene product inserts cytosines opposite apurinic/apyrimidinic sites (Nelson *et al.* 1996a), as one major source of AP sites is the depurination of G. The *RAD30* gene product is a DNA polymerase that inserts two As opposite of a cyclobutane thymidine dimer (Johnson *et al.* 1999). A more general translesion DNA polymerase is probably DNA polymerase ζ, which consists of two subunits encoded by *REV3* and *REV7* (Nelson *et al.* 1996b). These mechanisms are subsumed in a pathway that has been coined postreplicative repair, since stalling of DNA synthesis after DNA damage with successive release of the block has been observed (Rupp and Howard-Flanders 1968), and that has an error-

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prone and an error-free branch. Involved in this pathway are also ubiquitin-conjugating enzymes such as Rad6 (Jentsch *et al.* 1987) and Ubc13 (Hofmann and Pickart 1999), whose exact functions are not clear. One plausible role could be the removal of stalled replication or transcription complexes via ubiquitin-mediated proteolysis to allow access of the repair machinery to the damage.

Another source of mutations, which is independent of any chemical modification of DNA, is the misincorporation or insertion / deletion of nucleotides during replication. Mechanisms presently known to counteract such errors are the nucleotide selection step of the DNA polymerases, preferential exonucleolytic removal of misincorporated nucleotides at the replication fork by proofreading (Doublié and Ellenberger 1998; Roberts and Kunkel 1999), and postreplicative correction by DNA mismatch repair (Modrich and Lahue 1996) or by the recently discovered *RAD27*-dependent pathway (Tishkoff *et al.* 1997).

Organisms with more complex genomes require a higher replicational fidelity to maintain their genetic information than those with simpler genomes (Eigen and Schuster 1979), as is revealed, *e.g.*, by the low accuracy of reverse transcriptases from RNA viruses and the absence of DNA mismatch repair in several microorganisms as evidenced by the lack of the respective orthologues in their complete genomic sequences (Eisen 1998). On the other hand, the importance of these mechanisms for higher organisms is demonstrated by severe disorders elicited in humans through defects in certain repair systems. Well-documented examples are xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy, which are caused by defects in the nucleotide excision repair (Chu and Mayne 1996), and hereditary nonpolyposis colon cancer (HNPCC), which is due to inactive alleles of DNA mismatch repair genes (Modrich and Lahue 1996). The correlation between heritable human diseases and DNA repair makes the investigation of pathways with an impact on fidelity control important from a medical standpoint to define possible new determinants of such diseases. Here we describe the characterization of the new mutator gene *MPH1* from the yeast *S. cerevisiae* that also has homologs in mammals.

Mph1 has the seven signature motifs characteristic for the DEAH / DExH family of putative RNA helicases (Gorbalenya and Koonin 1993). Members of this protein family are numerous in both pro- and eucaryotes and fulfill a large variety of different cellular functions (Lüking *et al.* 1998). They have been shown to act in translation (Pause and Sonenberg 1992), splicing (Hamm and Lamond 1998), mRNA stability (Iost and Dreyfus 1994), transcription, UV repair (Deschavanne and Harosh 1993), and other processes involving nucleic acids. The function of several conserved motifs has been delimited by both mutational analyses (Pause and Sonenberg 1992; Pause *et al.* 1993; Gross and Shumann 1995, 1996) and the structure determination of the hepatitus C virus (HCV) RNA helicase domain (Yao *et al.* 1997). The Walker motif A (Walker *et al.* 1982; helicase motif I) is involved in binding of the terminal phosphate of the ATP, and the aspartate and glutamate of motif B (helicase motif II), which are part of the DExH sequence, are required for Mg^{2+} binding and hydrolysis. The histidine residue in this sequence may be involved in an NTP-dependent conformational change. The TAT sequence (helicase motif III) probably serves as a hinge region for rotation of the RNA-binding domain, which may be essential for helicase activity. RNA binding / unwinding requires the QRxGRxGR sequence (helicase motif VI), which presumably binds to single-stranded RNA via electrostatic interactions (Pause *et al.* 1993).

MATERIALS AND METHODS

Yeast strains and disruption plasmids: The *MPH1* disruption plasmid was constructed starting from the pBluescript II KS(+)-based plasmid pHI2 (source: A. Hinnen, Entian *et al.* 1999), which contained a *URA3* gene flanked by 5′ and 3′ adjacent chromosomal regions of *MPH1*. The *URA3* gene was deleted by cleavage with *Sma*I and *Cla*I, fill-in, and religation. Subsequently, the 3.8-kb *Bam*HI / *Bgl*II fragment from pNKY51 containing the *URA3* gene flanked by two direct repeats of the *Salmonella typhimurium hisG* gene (Alani *et al.* 1987) was inserted into the *Bam*HI site of the pHI2 derivative with the deleted *URA3* gene. The resulting plasmid, which was named pHI2-UH, contains the 5′ flanking region of *MPH1* from chromosomal coordinates 360398–361138 and the 3′ flanking region from 357405–356758. For *mph1* deletion construction, pHI2-UH was cleaved with *Pvu*II and used for transformation of strains CEN.PK2-1C**a** (*trp1-289 leu2-3,112 ura3-52 his3-*Δ*1 MAL2-8*^{c} *SUC2 MAT***a**; Entian *et al.* 1999) and MW3317-21A (Kramer *et al.* 1989). *ura3*$^{-}$ deletions were selected on 5-fluoroorotic acid (5-FOA; Sikorski and Boeke 1991), yielding strains CEN.PK2-1Cmph1Δ and MW3317-21Amph1Δ, respectively. The structure of the deletion was verified by PCR analysis. The mutator phenotype of CEN.PK2-1Cmph1Δ could be complemented by introduction of the centromere plasmid pRS313-MPH1, which contained the *MPH1* gene as a 4161-bp *Ssp*I-*Msc*I fragment (chromosomal coordinates: 360791–356631) inserted into the *Eco*RV-*Sma*I sites of pRS313 (Sikorski and Hieter 1989). Strains CEN.PK2-1C and CEN.PK2-1Cmph1Δ were transformed with a linear *URA3* gene fragment, isolated by *Pst*I-*Hin*dIII cleavage of plasmid pHI2-UH, to uracil prototrophy to yield strains CEN.PK2-1C-URA3^{+} and CEN.PK2-1Cmph1Δ-URA3^{+}, respectively. *hom3-10* derivates were constructed as described (Kramer *et al.* 1996). The presence of the *hom3-10* allele was verified by threonine auxotrophy and the increased reversion rate after additional deletion of *MSH2* (Reenan and Kolodner 1992; Lühr *et al.* 1998). *msh6*Δ mutants of CEN.PK2-1C were constructed as described (Lühr *et al.* 1998). The *mph1 msh6* double mutant was constructed by introducing the *msh6* deletion into CEN.PK2-1CmphΔ. Disruption mutants of CEN.PK2-1C and CEN.PK2-1Cmph1Δ for *mag1*, *apn1*, and *mms4* were constructed using the plasmids pJC8901 (*mag1::hisG-URA3-hisG*; Chen *et al.* 1990), pSCP19A (*apn1*Δ*::HIS3*; Ramotar *et al.* 1991), and pJWX1603 (*mms4*Δ*::hisG-URA3-hisG*; Xiao *et al.* 1998), which were obtained from W. Xiao. The disruption strains could be complemented with plasmids containing the respective wild-type genes, which were also obtained from W. Xiao. *rev3*, *rad6*,

rad14, *mms2*, and *rad52* deletion mutants were constructed by the method of Wach *et al.* (1994) using the kanMX4 module of plasmid pFA6 with PCR primers adding 40 nucleotides homology from the regions around the start and the stop codon at the ends of the kanamycin cassette. The PCR products were used to transform CEN.PK2-1C and CEN.PK2-1Cmph1Δ to G418 resistance. The deletion construction was verified by PCR analysis.

Nucleotide sequence of *ura3* mutations: Strains CEN.PK2-1C-URA3$^+$ and CEN.PK2-1C-mph1Δ-URA3$^+$, respectively, were streaked for single cells onto synthetic medium lacking uracil and 55 or 58, respectively, single-cell colonies were transferred individually onto 5-FOA plates (Sikorski and Boeke 1991). Single 5-FOA-resistant papilla were restreaked for single-cell colonies onto rich medium and one single-cell colony each was tested for *ura3* auxotrophy by lack of growth on synthetic medium without uracil and by complementation of the uracil auxotrophy by a plasmid-borne *URA3* gene. Chromosomal DNA from each thus characterized mutant clone was prepared (Gram positive bacteria & yeast DNA isolation kit, Puregene) and a 1237-bp fragment (chromosome V; 115917–117153) containing the *ura3* gene (116167–116967) was amplified by PCR (2 min denaturation at 95°; 30 cycles: 95° 45 sec, 55° 45 sec, 72° 90 sec; primers: 5′-TAATGTGGCTG TGGTTTCAGGGTC-3′ and 5′-TCTGGCGAGGTATTGGA TAGTTCC-3′) using *Tfl*-DNA-Polymerase (Promega, Madison, WI). The PCR products were purified with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and both strands were sequenced with a cycle sequencing kit (Amersham, Uppsala, Sweden) using IRD 800 labeled primers (MWG Biotech; forward, 5′-CTTAACCCAACTGCACAG-3′; nucleotide position on chromosome V, 116019; reverse, 5′-GAAGCT CTAATTTGTGAG-3′; nucleotide position on chromosome V, 117024). Sequences were analyzed on a LiCor DNA sequencer model 4000L.

Construction of the *MPH1-GFP* fusion gene: For construction of the *MPH1-GFP* fusion plasmid pRS316-GAL1-MPH1-GFP, first the *GFP5* fragment (Siemering *et al.* 1996) was PCR amplified using the primers gfp Sma: 5′-TCC CCC GGG ATG GCT AGC AAA GGA GAA GAA C-3′ and 5′-ATG CCT GCA GGT CGA CTC TAG AGG-3′, which introduced an additional *Sma*I site at the 5′ end of the gene. The PCR fragment was digested with *Sma*I and cloned into the *Swa*I site of plasmid pRS316-GAL1-MPH1. This plasmid contains the *MPH1* gene under control of the *GAL1* promoter. The *Swa*I site was introduced at the very end of the *MPH1* gene changing the second last codon from an aspartic acid codon to a glutamic acid codon and introducing an additional lysine codon before the stop codon. (Details of the constructions are available on request from the authors.) Furthermore, a *Sma*I/*Fsp*I fragment from pRS316-GAL1-MPH1-GFP, carrying the *MPH1-GFP* fusion gene, was cloned into the *Ecl*136II site of plasmid pYES2 (Invitrogen, Carlsbad, CA) to yield plasmid pYES2-MPH1-GFP. Control plasmids expressing *GFP* alone were constructed by cloning the *Sma*I-digested PCR-amplified *GFP* fragment (see above) into the *Sma*I site of pRS316-GAL1 or into the *Ecl*136II site of pYES2, yielding the plasmids pRS316-GAL1-GFP and pYES2-GFP, respectively.

For delimiting the nuclear localization signal of Mph1, a *Hinc*II fragment of *MPH1* coding for amino acids 675–838 was cloned into the *Ecl*136II site of pYES-GFP, located 9 bp upstream of the stop codon of the GFP5 gene. Using the primers gfp-Sma (see above) and 5′-GCT CTA GAT TTT TTT GGC TTC ACC TT or 5′-GCT CTA GAA ACT CTC TTC TTC TTT GT as primers at the 3′ end of the gene, fragments of the fusion gene containing *MPH1* segments coding for amino acids 675–696 or 675–704, respectively, were amplified where an *Xba*I site was introduced *via* the 3′ primers. The PCR fragments were cleaved with *Msc*I/*Xba*I (*Msc*I is an internal site in the GFP gene) and cloned into the corresponding sites of pYES-GFP.

Transformants of CEN.PK2-1C with these plasmids were grown for 24 hr in synthetic medium lacking uracil with 2% galactose as a carbon source before evaluating the fluorescence with an Axioskop fluorescence microscope (Zeiss, Oberkochen, Germany). For identification of nuclei, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml) after brief fixation in 40% ethanol/0.1 m sorbitol. For gfp fluorescence Zeiss filter set 487709 was used and for DAPI fluorescence Zeiss filter set 487702 was used.

Mutation rates: Mutation rates were determined according to the method of the median (Lea and Coulson 1948) with 11 parallel cultures unless otherwise indicated. Five-milliliter cultures were inoculated with ~100–1000 cells and grown at 30° for 3 days in YPD or, for plasmid-containing strains, in selective synthetic medium and aliquots were plated onto selective plates. For strains containing plasmids, the plates were also selective for the plasmid marker. The titer in the cultures was counted with a hematocytometer or, for the epistasis analyses with *rad6*, *rad14*, *mms2*, and *rad52*, the viable titer was determined. Heteroduplex-DNA repair assays were as described (Lühr *et al.* 1998).

Sensitivity against chemicals and radiation: Methyl methanesulfonate (MMS) and 4-nitroquinoline 1-oxide (4-NQO) were purchased from Fluka Chemical (Buchs, Switzerland); 6-azauracil, hydroxyurea, and camptothecin were from Sigma (St. Louis). Sensitivity tests were performed on plates by growing an overnight culture of the respective strain in liquid YPD at 30°, adjusting the cell density, and spotting serial 1:10 dilutions onto SC plates containing 60 μg/ml 6-azauracil or onto YPD plates containing 100 mm hydroxyurea or MMS, EMS, 4-NQO, or camptothecin at the concentrations indicated. Plates were incubated at 30° for 2–4 days.

For MMS survival curves, overnight cultures of the respective strains were diluted into YPD to a cell density of 1×10^6 cell/ml and grown with shaking to a density of 1×10^7 cell/ml. An aliquot was removed for determination of the viable titer and MMS was added to a final concentration of 0.3%. Samples were withdrawn after 20, 40, and 60 min, the cells were washed with YPD, and appropriate dilutions were plated onto YPD plates to determine the viable titer.

UV survival curves were recorded by growing cells as described above for MMS killing curves to a density of 1×10^7 cells/ml. Cells were washed and resuspended in water at a density of 1.5×10^7 cell/ml. Cells were irradiated with shaking with a noncalibrated UV lamp (UV-C range, burner Osram HNS10W, distance ~30 cm). Aliquots were withdrawn at different times, diluted, and plated onto YPD plates to determine the number of survivors after incubation at 30°. For semiquantitative plate tests, 4×10^6 cells of overnight cultures in YPD were streaked across a square YPD plate (12 × 12 cm) and segments of the streaks were irradiated for various times. Plates were incubated in the dark at 30° for 2 days.

For X-ray survival curves, overnight cultures in YPD were washed and resuspended in 100 mm sodium-potassium phosphate buffer, pH 7.2. Cells were irradiated with agitation with a calibrated X-ray source (200 kV, 20 mA, filter 0.5 Cu; Siemens) for different doses. Aliquots were withdrawn and dilutions were plated onto YPD plates to determine the number of survivors after incubation at 30°.

RESULTS

In a systematic approach to analyze the function of unknown open reading frames of *S. cerevisiae* discovered during the yeast genome sequencing project, mutants with a deletion of open reading frame *YIR002c* were

found to have an increased spontaneous mutation rate in a forward mutation assay to canavanine resistance and in a reversion assay of the *amber* mutation *trp1-289* (CAG → TAG; codon 130 of *TRP1* gene). The mutant did not display any pleiotropic phenotype in other tests employed in the functional analysis network such as growth on different carbon sources (glucose, galactose, mannose, fructose, ethanol, acetate, glycerol), formation of *rho*$^-$ cells, distribution of cells in different stages of the cell cycle, sensitivity to various stresses (H_2O_2, NaCl, KCl, $CuSO_4$, temperature), changes in morphology and cytoskeleton (bud scars, nucleus, mitochondria, vacuole, actin, tubulin), mating, invasive growth, glycosylation and secretion, plasmid loss and sensitivity to thiabendazole, and UV-induced mutations (Entian *et al.* 1999).

YIR002, which had been renamed *MPH1* (*m*utator *ph*enotype; Entian *et al.* 1999), is located on the right arm of chromosome IX (position 357415–360393) and could code for a protein of 993 amino acids. According to the classification given by Gorbalenya and Koonin (1993), Mph1 belongs to the DEAH family in the superfamily 2 (SF2) of helicases, since it contains all seven characteristic motifs (motif I, PTGMGKT; motif Ia, PTRPLV; motif II, VIDEAH; motif III, ALTAT; motif IV, RVIIFT; motif V, TSIGEEGLDI; motif VI, QRMGRTGR).

For the present study, we have first determined the transcription of the *MPH1* gene and the meiotic behavior of mutants. It was shown by reverse transcription (RT)-PCR (Kramer *et al.* 1996) that transcription is not cell cycle regulated and is not induced during meiosis (data not shown). Some induction (~3.8-fold) has been reported after treatment with MMS (Jelinsky and Samson 1999). Homozygous *mph1* diploids displayed a slight sporulation defect. Starting from isolated zygotes, wild-type cells produced 30% four spore asci (78 asci from a total of 255 cells), whereas the homozygous *mph1* mutant yielded only 8% four spore asci (19 asci from a total of 256 cells). Spore survival, however, which is a sensitive indicator for several disturbances during sporulation, was not reduced. After tetrad dissection, 89.6% viable spores were observed in homozygous *mph1* diploids (190 survivors / 212 spores) and 92.4% in wild type (207 survivors / 224 spores; three isolated zygotes each). It can be concluded that *mph1* mutants do not have a severe meiotic defect.

Possible indirect effects and nuclear localization: Members of the DEAH family are often associated with RNA metabolism (Lüking *et al.* 1998). The mutator effect of *mph1* mutants could therefore be due to an indirect effect, *e.g.*, that lack of the *MPH1* gene product may reduce the translational efficiency of a protein involved in fidelity control or may not allow efficient splicing of an intron containing pre-mRNA. To investigate this in more detail, the subcellular localization of the *MPH1* gene product was determined. The gene for green fluorescent protein (*gfp*) was fused to the 3′ end of the open reading frame of *MPH1* and the fusion gene was expressed from a multicopy plasmid under control of the *GAL1* promoter. The *MPH1-gfp* fusion gene (with the natural *MPH1* promoter) was able to complement the mutator phenotype of an *mph1* mutant (data not shown). As can be seen in Figure 1A, the Mph1-gfp derived fluorescence colocalizes with the DAPI fluorescence indicative for the nucleus. Expressing gfp without the Mph1 moiety resulted in uniform staining of the cell.

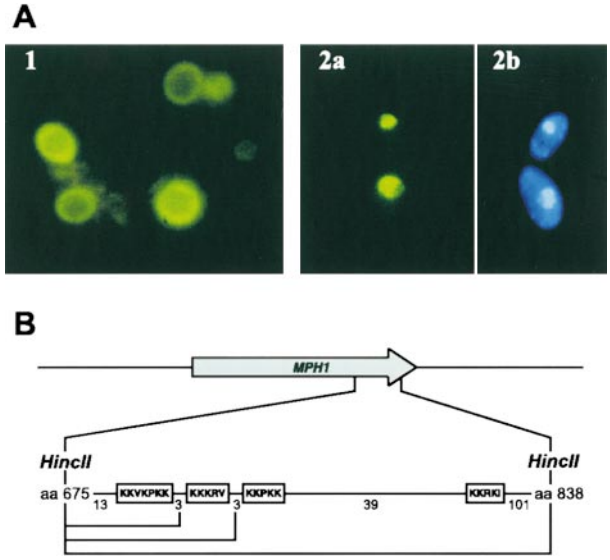

Figure 1.—Nuclear localization of Mph1. (A) Fluorescence microscopy. (1) CEN.PK2-1C transformed with pRS316-GAL1-GFP expressing gfp alone. The filter set was Zeiss 487709 for observing gfp fluorescence. (2a) CEN.PK2-1C transformed with pYES2-MPH1-GFP expressing a Mph1-gfp fusion protein under control of a *GAL1* promoter. Cells were also stained with DAPI. The filter set was Zeiss 487709. (2b) Same cells as in 2a, but with filter set Zeiss 487702 for DAPI fluorescence. (B) Schematic drawing of putative nuclear localization signals in Mph1. The enlarged *Hin*cII fragment codes for amino acids 675–838. The brackets indicate the fragments expressed as C-terminal fusion with gfp for delimitation of the minimal sequence requirements to direct nuclear localization. The numbers in small type are the distances in amino acids between the elements shown.

Inspection of the Mph1 amino acid sequence revealed several clusters of basic amino acids between positions 675 and 838 (Figure 1B), which are typical for nuclear localization signals. To investigate whether sequences in this region are sufficient for directing nuclear localization, several peptides from this region as shown in Figure 1B were produced as C-terminal fusion with gfp. As judged from fluorescence microscopy, all peptides were able to direct localization of gfp to the nucleus (data not shown).

The nuclear localization of Mph1 largely excludes indirect effects on translation. To test for possible defects in splicing, total RNA from wild-type and *mph1*

mutant strains was prepared and the amounts of spliced and unspliced mRNA for *MMS2*, *UBC13*, *UBC4*, and *ACT2* were determined by RT-PCR (Kramer *et al.* 1996) using PCR primers flanking the intron. *MMS2* (Broomfield *et al.* 1998) and *UBC13* (Hofmann and Pickart 1999) were chosen, since the phenotypes of *mms2* and *ubc13* mutants have some resemblance to the phenotype of *mph1* mutants (see below) and thus a splice defect might result in phenocopies of the respective mutants. No significant increase in the amount of unspliced mRNA was observed in any of the four genes in the *mph1*-derived RNA preparation compared to wild type (data not shown).

To test whether the conserved motifs in Mph1 are necessary for its function in fidelity control, single-amino-acid substitution mutations were constructed in motif I (K113Q), motif II (D209N, E210Q, H212D), and motif VI (Q603D). The mutant genes were introduced on a centromere plasmid into *mph1* mutants and tested for complementation. None of the mutant genes was able to complement the mutator phenotype of the *mph1* mutant (data not shown). To exclude the possibility that the mutant proteins produced were unstable, fusions of the mutant proteins with gfp were constructed. As judged from fluorescence microscopy, all fusion proteins were present in similar amounts and were localized to the nucleus (data not shown).

Mutation spectrum: To possibly delimitate the function of *MPH1*, the spectrum of spontaneous mutations in a wild-type strain and an isogenic *mph1*Δ mutant was recorded. In case a particular type of mutation would occur predominantly in *mph1* mutants, important clues to the possible function could be obtained. First, the *ura3-52* alleles in CEN.PK2-1C and CEN.PK2-1Cmph1Δ were replaced by transformation with a functional *URA3* gene. To select for spontaneous uracil auxotrophic mutants, 55 or 58 uracil prototrophic single-cell colonies from wild type or mutant, respectively, were streaked onto 5-FOA. One mutant colony derived from each single-cell colony was restreaked for single-cell colonies; the *ura3* gene from one colony was amplified by PCR and sequenced. This procedure ensured that all mutations were independent events. The results of this analysis are summarized in Table 1.

The *ura3* mutations both in wild type and in *mph1* mutants were more or less evenly distributed over the entire length of the gene with no obvious hotspots (data not shown). It can thus be concluded that *MPH1* does not exert a strong preference for a particular sequence context. Also, no strong preponderance of a particular type of mutation occurring in the *mph1* mutants was observed compared to wild type (Table 1). The conspicuous disparity in transition mutations (A → G *vs.* G → A and C → T *vs.* T → C) is similar in wild type and *mph1* mutants and thus most likely constitutes an intrinsic property of the experimental system, since it has also been observed in other mutation spectra using *URA3*

TABLE 1

Summary of the spontaneous URA3 mutation spectrum in a wild-type and a *mph1* mutant strain

	Wild type	*mph1*
Transversions		
A·T→T·A	9	13
A·T→C·G	4	3
G·C→T·A	8	12
G·C→C·G	3	6
Σ	24	34
	(44%)	(59%)
Transitions		
A·T→G·C	5	3
G·C→A·T	15	18
Σ	20	21
	(36%)	(36%)
Deletions/insertions		
−A	5	0
−G	2	1
+GG	1	0
Σ	8	1
	(15%)	(2%)
Duplications		
+CAAA	2	0
+46-bp	1	0
Σ	3	'0
	(5%)	(0%)
Rearrangements		
	0	TAAAAACACA→A_{14}
	0	CTGATTT→CTAATT
Σ	0	2
	(0%)	(3%)
Σ	55	58

Mutation spectra were recorded using the isogenic strains CEN.PK2-1C-URA3$^+$ and CEN.PK2-1C-mph1Δ-URA3$^+$. For insertions, deletions, and rearrangements, the changes occurring on the coding strand are indicated. For base substitutions, the classes comprise mutations where the first nucleotide of an indicated base pair was on the coding strand, as well as mutations where the first nucleotide was on the noncoding strand.

(Kunz *et al.* 1998). There is, however, an underrepresentation of one or two nucleotide insertions/deletions in the *mph1* mutant (one in *mph1* mutant, eight in wild type).

To gather further evidence for the conjecture derived from the mutation spectrum that frameshift mutations are not increased in *mph1* mutants, the reversion rate of the *hom3-10* allele was determined. *hom3-10* is a T addition in a run of six Ts (Marsischky *et al.* 1996). The reversion rate of *hom3-10* in a *mph1* background was 7.5×10^{-9} *vs.* 7.8×10^{-9} in the isogenic wild-type strain and thus not increased in the mutant, consistent with the interpretation of the mutation spectrum. The forward mutation rate to canavanine resistance of the *mph1* mutant was increased eightfold compared to wild type in this experiment (3.7×10^{-7} *vs.* 4.5×10^{-8}).

The predominance of base substitutions *vs.* inser-

tion/deletions in *mph1* mutants resembles the mutation spectrum of an *msh6* mutant, whereas another DNA mismatch repair mutant, *msh2*, showed a strong predominance of frameshifts over base substitutions (Marsischky *et al.* 1996). To see whether *MPH1* acts in a *MSH6*-dependent pathway, an epistasis analysis of the mutator phenotypes was carried out. The forward mutation rate to canavanine for the *mph1* mutant was increased 12-fold compared to wild type (1.3×10^{-6} *vs.* 1.1×10^{-7}) and for the *msh6* mutant 25.5-fold (2.8×10^{-6}). The mutation rate of the *mph1 msh6* double mutant was elevated 35.5-fold (3.9×10^{-6}) and is thus additive. It can therefore be concluded that *MPH1* is not part of the *MSH6*-dependent branch of DNA mismatch repair.

MPH1 might nevertheless be involved in DNA mismatch repair having some more general function. In *E. coli*, the DNA helicase UvrD is required for fully efficient mismatch repair (Modrich and Lahue 1996). To test this in more detail, an *mph1* mutant was transformed with heteroduplex DNA with defined mismatches (G/T, G/G, A/Δ, C/C) in an assay as described in Lühr *et al.* (1998). No obvious difference in the repair of the selected mismatches could be observed between *mph1* mutants and wild type (data not shown). Therefore, it can be concluded that *mph1* is likely not involved in DNA mismatch repair.

Sensitivity of *mph1* mutants against DNA-damaging treatments: A variety of mutants affected in several pathways for repair of premutagenic lesions show a spontaneous mutator phenotype. Therefore, the sensitivity of *mph1* mutants against various physical treatments or chemicals damaging DNA or otherwise interfering with nucleic acid metabolism was tested (UV light, X rays, MMS, EMS, 4-NQO, camptothecin, hydroxyurea, 6-azauracil). Far UV light induces mainly bulky lesions such as cyclobutane pyrimidine dimers and 6-4-photoproducts (Cadet 1994). X rays induce double-strand breaks both via direct absorption and radicals produced by radiolysis, which also cause a whole spectrum of other damages (Hutchinson 1985; Ward 1988). MMS and EMS (ethyl methanesulfonate) are both alkylating agents transferring methyl and ethyl groups, respectively, to nucleophilic centers in DNA thus forming a variety of different adducts. The second most frequent MMS adduct after N^7-methylguanine is the toxic N^3-alkyladenine (Pegg 1984), which blocks replication. O^6-alkylguanine is probably more prominent among the EMS adducts (Drake and Baltz 1975; Singer 1975). After metabolic activation, 4-NQO forms bulky adducts with nucleobases (Turesky 1994), which are primarily targets for nucleotide excision repair. Therefore, 4-NQO is sometimes referred to as UV mimeticum, although it produces a significant amount of oxidative damage as well (Ramotar *et al.* 1998). Camptothecin is not a DNA-damaging agent by itself, but traps the covalent topoisomerase I/DNA intermediate (Pommier *et al.* 1998), which could lead to double-strand breaks during replication (Liu *et al.* 1996). Hydroxyurea reduces the amount of dNTPs thus inhibiting DNA synthesis, and 6-azauracil reduces the UTP and GTP pools thus interfering with transcription (Hampsey 1997).

It was found that *mph1* mutants are, if at all, only very slightly sensitive to X rays (Figure 4) and to UV light (Figure 5 and data not shown). They are also not sensitive to hydroxyurea and 6-azauracil (data not shown). They are, however, sensitive to MMS, EMS, 4-NQO, and camptothecin (Figures 2 and 3). This sensitivity is most likely not due to a defect in the G2/M DNA damage checkpoint (Paulovich *et al.* 1997), since the *mph1* mutant did show G2 arrest (as judged microscopically from the accumulation of dumbbell cells) at least as efficiently as wild type after challenging cells with MMS (data not shown). This argues for an involvement of *MPH1* in a pathway protecting the genome against lesions caused by these chemicals, either by preventing or repairing these lesions. It is particularly noteworthy that *mph1*-mutants are quite sensitive to 4-NQO but not to UV light.

***REV3* dependence of *mph1* mutator phenotype:** The mutator phenotype of many DNA repair mutants results from channeling unprocessed or partially processed DNA lesions into the error-prone pathway of postreplicative repair (PRR), which results in the mutagenic bypass of these lesions by the Rev3/Rev7 DNA polymerase (Nelson *et al.* 1996b). Therefore, *mph1* double mutants with *rev3* were constructed and tested for spontaneous mutator phenotype in a canavanine forward mutation assay. The mutator phenotype of *mph1* is abolished in *mph1 rev3* double mutants (mutation rates: wild type, 1.5×10^{-7}; *rev3*, 1.0×10^{-7}; *mph1*, 1.5×10^{-6}; *mph1 rev3*, 1.7×10^{-7}). This result indicates that the mutator phenotype of *mph1* mutants results from premutagenic lesions that are processed by error-prone PRR and that Mph1 is not required for enhancement of fidelity during replication.

Interaction with known repair pathways: The sensitivity of *mph1* mutants to various DNA-damaging chemicals and the dependence of the spontaneous mutator phenotype on *REV3* raises the question whether *MPH1* is part of a known repair pathway. To investigate this, an epistasis analysis of *mph1* mutants with mutants in base excision repair, nucleotide excision repair, postreplicative repair, and recombinogenic repair was carried out.

As representatives for base excision repair, *MAG1*, encoding a wide substrate specificity glycosylase removing alkylated adenines (Chen *et al.* 1990), and *APN1*, encoding the major AP-endonuclease in yeast (Popoff *et al.* 1990), were selected. Furthermore, interaction with *MMS4*, which was identified by virtue of the MMS sensitivity of mutants (Xiao *et al.* 1998), but has no clearly assigned function yet, was tested. MMS-induced killing curves for the various single and double mutants were recorded (Figure 2).

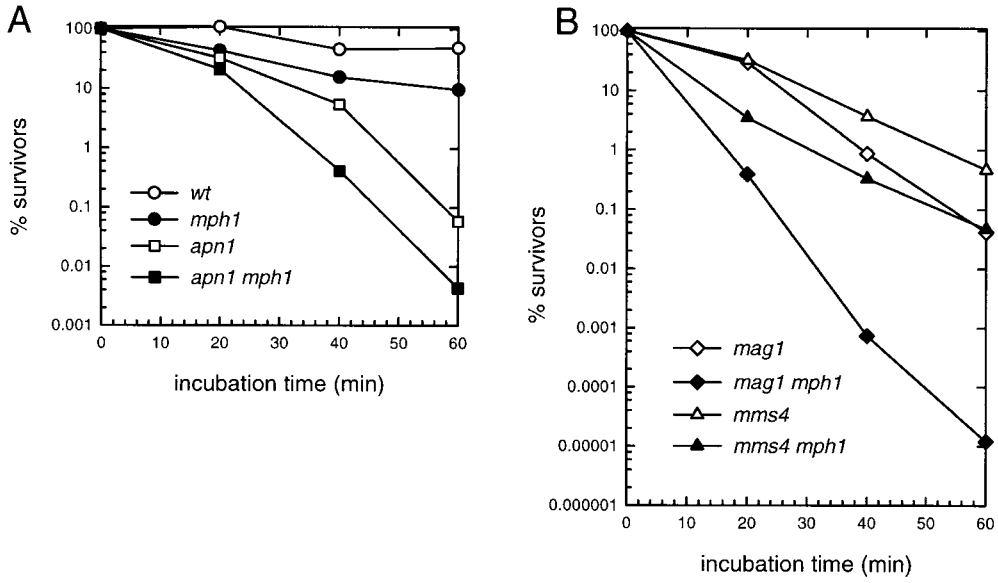

Figure 2.—MMS-induced killing in various mutant strains. Isogenic derivatives of strain CEN.PK2-1C with the relevant genotypes, as indicated in the figure, were incubated with 0.3% MMS. Aliquots were withdrawn at the indicated times, the viable titer was determined, and the percentage of survivors was calculated. The percentage of survivors was plotted on a logarithmic scale. The data were distributed onto two plots, A and B, for clarity. Scales are identical for plots A and B. The data are the average of two independent experiments.

The *mph1* mutant is the least sensitive of all single mutants, followed by *mms4*, *apn1*, and *mag1*. All double mutants with *mph1* were more sensitive than the respective single mutants, with *mag1 mph1* being the most sensitive. The lack of epistasis in the double mutants argues for an involvement of *MPH1* in a repair pathway different from the *MAG1 APN1*-dependent base excision repair pathway of alkylated adenines.

As representative for nucleotide excision repair *RAD14*, for recombinogenic repair *RAD52*, for both error-prone and error-free PRR *RAD6* and for the error-free branch of PRR *MMS2* and the error-prone branch *REV3* was chosen. The *rev3*, *mms2*, *rad6*, *rad14*, and *rad52* single mutants and the respective double mutants with *mph1* were tested in a drop dilution plate assay for sensitivity against MMS, EMS, 4-NQO, and camptothecin. As can be seen in Figure 3, all *mph1* double mutants are more sensitive than the respective single mutants, with the possible exception of the *mph1 rad52* mutant on camptothecin. Since the sensitivities of the *mph1* mutant are not epistatic to any of the repair mutants tested, it can be concluded that *MPH1* is not involved in one of the known major repair pathways.

The above mutants were also tested for X-ray and UV light sensitivity. None of the double mutants with *mph1* was more sensitive to X rays than the respective single mutants (Figure 4). UV light sensitivity was tested in a semiquantitative plate assay. As can be seen in Figure 5, double mutants of *mph1* with *mms2*, *rad6*, and *rad14* were slightly more sensitive to UV light than the respective single mutants.

To investigate whether an overlap exists for the premutagenic lesions leading to mutations in *mph1* mutants and other repair mutants, an epistasis analysis for the spontaneous mutation rates to canavanine resistance was carried out with the mutants shown in Table 2. Mutation rates were determined by the method of the median (Lea and Coulson 1948). The data for *rev3* confirms the data obtained before. The reduction of the mutation rate observed in *rad6 mph1* compared to *mph1* is expected, if the spontaneous mutations in *mph1* mutants arise via the error-prone pathway of PRR, since *rad6* is required for this pathway as well. The mutation rates for *rad52* and *mph1* are additive, indicating that the mutations arise from different kinds of premutagenic lesions. The mutation rates of *mms2* and *mph1* are lower than additive, but also not epistatic, whereas the mutation rates of *rad14* and *mph1* seem to be more than additive, but also not clearly synergistic. A simple interpretation of this behavior is difficult as long as neither the nature of the lesions leading to mutations in particular mutants nor the exact role of the genes is defined. But as for the other tests, no clear epistatic interaction of *mph1* with any of the other mutants could be observed.

Mph1 homologs: A BLAST search comparing the deduced amino acid sequence of *MPH1* with the databases (Altschul *et al.* 1997) gave as the closest homologs putative proteins from the archaea *Archaeoglobus fulgidus*, *Methanococcus janaschii*, *Methanobacterium thermoautotrophicum*, *Pyrococcus abyssii*, *P. horiskoshii*, and *Cenarchaeum symbiosum* with E values between $3 \times e^{-50}$ and $3 \times e^{-21}$. They had identical residues at 37% of 601 aligned amino acids, 34% of 646, 32% of 570, 30% of 678, 33% of 551, and 32% of 544, respectively. No close homolog that might be considered to be an ortholog was found in completely sequenced genomes of eubacteria or *Caenorhabditis elegans*. Among the list of proteins with significant sequence homology were also matches to the transcription repair coupling factors of eubacteria and the proteins from the RecQ family, which include the yeast gene *SGS1* (Watt *et al.* 1995) and the human disease genes *BLM* and *WRN*, which are required for genome stability (German 1993; Epstein *et al.* 1966). A multiple sequence alignment of RecQ, Blm, Sgs1, and

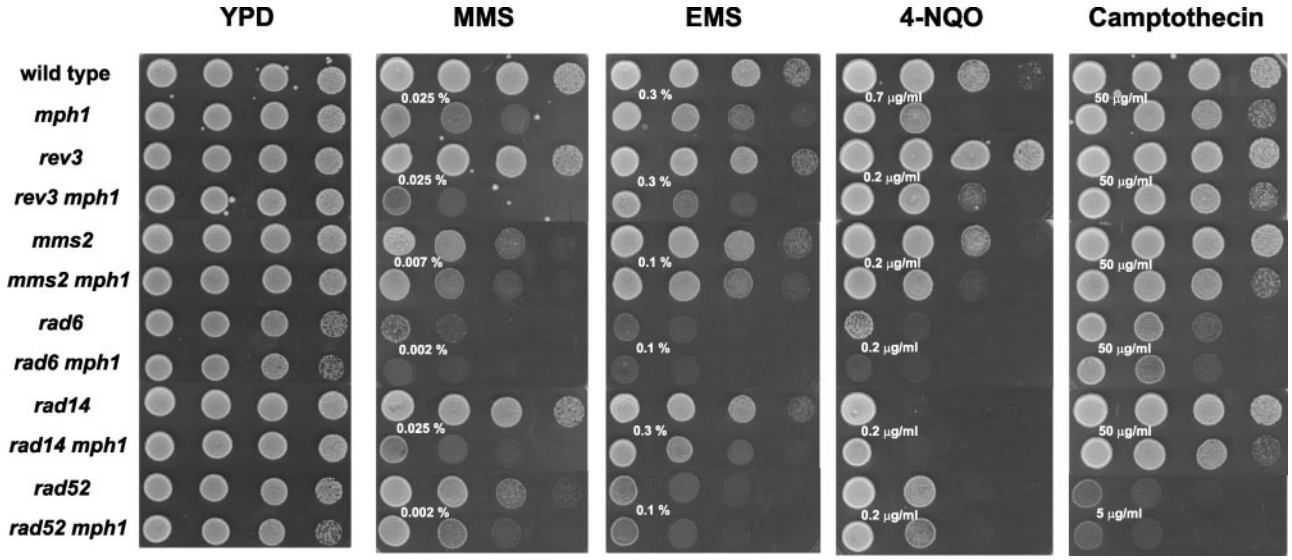

Figure 3.—Sensitivity of various mutants to MMS, EMS, 4-NQO, and camptothecin. Isogenic derivatives of strain CEN.PK2-1C with the relevant genotypes, as indicated to the left of the figure, were spotted onto plates containing the chemicals, as indicated on top. The spots contained, from left to right, 10^5, 10^4, 10^3, and 10^2 cells, respectively. The YPD plate served as growth control. Each mutant and its respective *mph1* double mutant were analyzed on the same plate. The concentration of the chemical in a particular plate is given in white letters between the respective pair.

Mph1, where the conserved boxes were forced to align, showed 18.5% of the amino acids that were identical in Mph1 with either of the three proteins over an alignment length of 940 amino acids (data not shown). Mph1 is, however, not a member of the RecQ familiy, *e.g.*, due to sequence differences in motif I, which are shared by all RecQ family members.

Comparing the deduced amino acid sequence of *MPH1* with the expressed sequence tag (EST) libraries held at the National Center of Biotechnology Information yielded hits with human, mouse, and *Drosophila melanogaster* ESTs. A multiple sequence alignment of the deduced amino acid of these clones with that of *MPH1* is shown in Figure 6. The extensive homologies, which are also found outside of the conserved boxes, strongly suggest that these ESTs represent orthologous genes to *MPH1*.

DISCUSSION

In this study, we have characterized the new mutator gene *MPH1* from *S. cerevisiae*. The deduced amino acid sequence of this gene reveals the key signature motifs characteristic for the DEAH family of RNA helicases. After determination of the structure of such a helicase from hepatitis C virus (Yao *et al.* 1997) the functions of many of the conserved motifs assigned previously by mutational analyses could also be explained on a structural basis (see also Introduction). Since we have demonstrated by introducing mutations into the conserved motifs that these motifs are necessary for the function of Mph1 in fidelity control and that this function does not reside in a different domain of the protein, the possibility of Mph1 being an RNA helicase has to be considered.

The effect of *MPH1* is probably not exerted indirectly: The mutator phenotype of *mph1* mutants can be elicited in two principal ways: either Mph1 partakes directly in a mechanism safeguarding genetic informa-

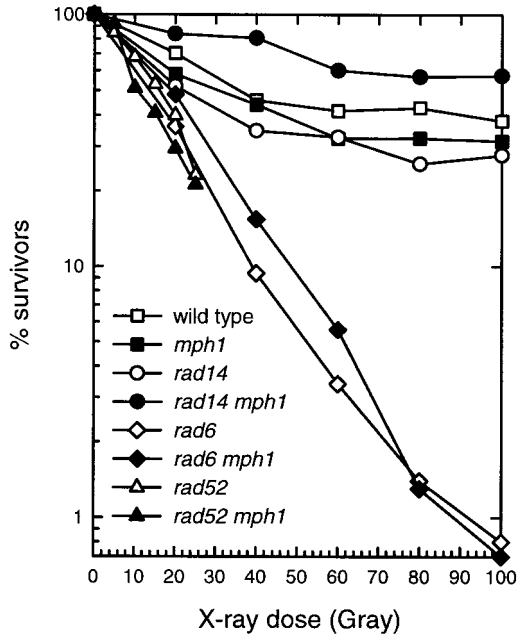

Figure 4.—X-ray sensitivity of various mutant strains. Isogenic derivatives of strain CEN.PK2-1C with the relevant genotypes, as given in the figure, were irradiated with X rays at the doses indicated. The viable titer was determined and the percentage of survivors was calculated. The percentage of survivors was plotted on a logarithmic scale.

TABLE 2

Spontaneous mutation rates to canavanine resistance for several DNA repair mutants

Mutant	Mutation rate ($\times 10^7$)	Factor
Wild type	1.2	1
mph1	14.8	12
mms2	16.1	13
mms2 mph1	22.6	18
rad6	3.2	2.7
rad6 mph1	6.1	5
rev3	0.57	0.5
rev3 mph1	1.8	1.5
rad14	5.4	4.5
rad14 mph1	36.3	30
rad52	19.4	16
rad52 mph1	38.1	32

Isogenic derivatives of strain CEN.PK2-1C with the relevant genotypes as given were grown in rich medium and mutation rates were determined by the method of the median (Lea and Coulson 1948) using nine parallel cultures for each strain.

tion or the effect is exerted indirectly. In accordance with previously described activities of RNA helicases, one might envision a function of Mph1 in translation or splicing. Defects in these processes might lead to a reduction in the amount of proteins directly required for fidelity control. A defect in translation, however, can be largely excluded, since we demonstrated using a fusion with gfp that the protein is transported into the nucleus. A defect in splicing is also unlikely, since we found that four different RNAs tested were spliced not notably less efficiently in the *mph1* mutant than in wild type. It cannot, however, be rigorously excluded that Mph1 might specifically affect splicing of another RNA whose gene product is involved in fidelity control or participates in other nuclear processes involving RNA such as nuclear export or transcriptional fidelity. In this case, however, one would expect the *mph1* mutants to have a pleiotropic phenotype.

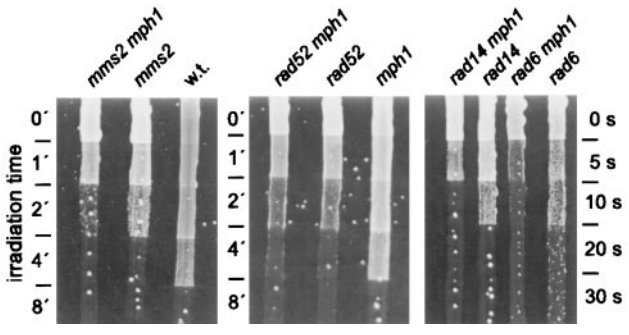

Figure 5.—UV sensitivity of various mutant strains. Isogenic derivatives of strain CEN.PK2-1C with the relevant genotypes, as given on top of the figure, were streaked onto plates and irradiated with a germicidal UV lamp. Irradiation times for the different segments are minutes for the left two panels and seconds for the rightmost panel. The distance of the lamp was the same for all three plates.

***MPH1* is probably not a component of an established repair pathway:** Since an indirect effect of the Mph1 protein is unlikely, a more immediate involvement in DNA metabolism has to be considered. The most prominent examples of DEAH helicases with DNA helicase activity are the members of the RecQ family such as RecQ from *E. coli* (Kowalczykowski *et al.* 1994), Sgs1 from *S. cerevisiae* (Watt *et al.* 1995), and the human Bloom and Werner proteins, which are lacking in patients with the respective diseases (German 1993; Epstein *et al.* 1966). Although we did not succeed in a first attempt with partially purified Mph1 protein to demonstrate DNA helicase- or DNA- (or RNA-) dependent ATPase activity, the homologies nevertheless suggest that Mph1 might have such an activity.

Taking this aspect into consideration opens up a multitude of new prospects for a possible function of Mph1. A number of established repair pathways are known to require helicases or the necessity of helicase activity can be readily imagined. In an attempt to narrow the breadth of possibilities, we have carried out several epistasis analyses. An involvement of Mph1 in DNA mismatch repair or other mechanisms improving replicational fidelity can be largely excluded. The mutator phenotype of *mph1* is additive to that of *msh6*, a gene involved in DNA mismatch repair. Furthermore, no defect in the repair of heteroduplex DNA with defined mismatches was observed in a *mph1* mutant. The dependence of the mutator phenotype of *mph1* on *REV3*, which encodes a subunit of the polymerase ζ required for error-prone translesion synthesis (Nelson *et al.* 1996b), makes participation in any process that increases fidelity at the replication fork very unlikely, since most or all of the mutations in *mph1* mutants seem to arise by mutagenic bypass of unrepaired lesions. But a participation in base excision, nucleotide excision, error-prone, and error-free postreplicative repair and recombinational repair can be largely excluded also, since representative mutants from all these pathways (*mag1*, *apn1*, *rad14*, *rad6*, *rev3*, *mms2*, and *rad52*) are less sensitive to chemical treatment than the respective double mutants with *mph1*.

Possible functions of *MPH1*: Thus, Mph1 is most likely not part of a hitherto known repair pathway. This raises the question of other possible functions. Three observations seem to be particularly valuable to arrive at a plausible speculation: (i) The mutation spectrum of *mph1* is very similiar to wild type. The only obvious difference is the underrepresentation of frameshift mutations. (ii) The lack of *MPH1* increases the sensitivity to various chemicals for mutants from all repair pathways, *i.e.*, base excision repair, nucleotides excision repair, error-free and error-prone postreplicative repair, and recombinational repair. (iii) *mph1* mutants are not or only slightly sensitive to UV irradiation, but they are quite sensitive to the UV mimeticum 4-NQO.

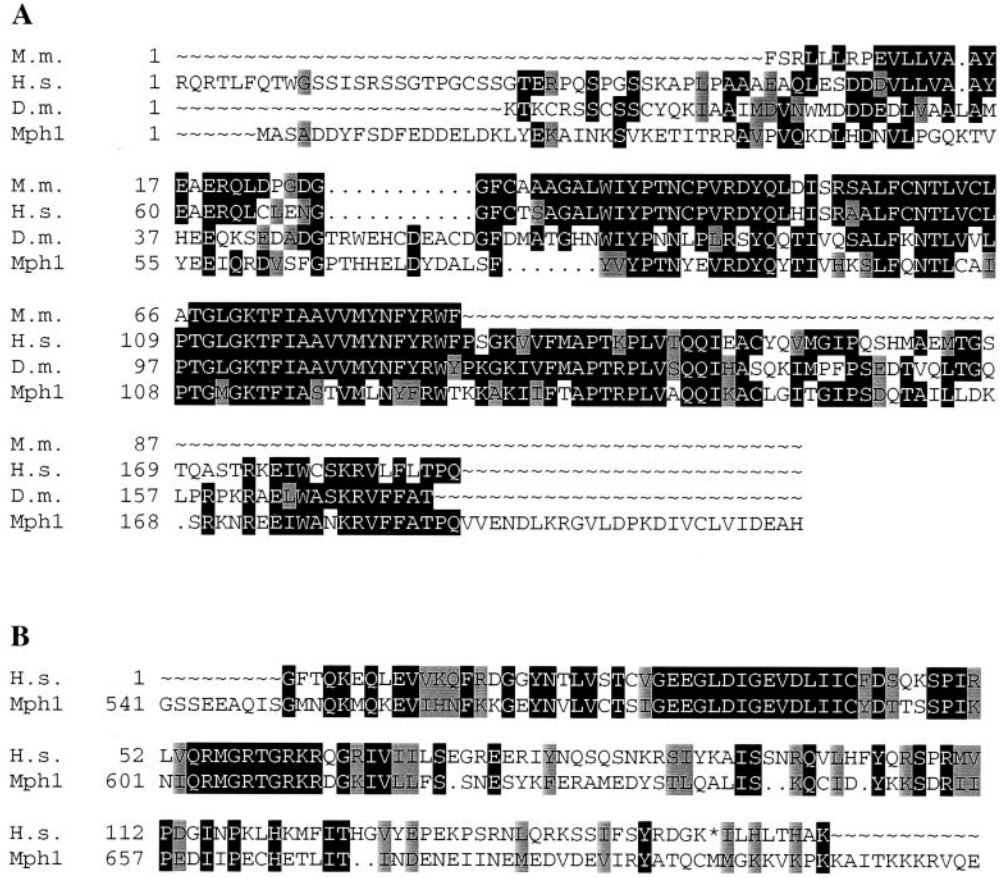

Figure 6.—Alignment of deduced amino acid sequences of several EST clones with that of *MPH1*. Only the translated regions of the EST clones with homology to Mph1 are shown. Sequences were aligned with the "Pileup" program of the GCG program package of the University of Wisconsin using the Blosum62 matrix. The multiple sequence alignment was then processed with the "Boxshade" program (http://www.ch.embnet.org/software/BOX_form.html) to highlight identities and similarities (fraction for highlighting 0.5). Identities are highlighted with a solid background, similarities with a shaded background. The abbreviations in the alignments refer to the following EST clones: (A) M.m., *Mus musculus* clone 445961; H.s., *Homo sapiens* clone 731587; D.m., *D. melanogaster* clone ld47993; Mph1, Mph1 from *S. cerevisae*. (B) H.s., *Homo sapiens* clone 731541; Mph1, Mph1 from *S. cerevisae*. For the mouse clone, it was demonstrated by both PCR and Southern blot analysis that the respective sequence is present in the mouse genome, excluding the possibility of a contamination (data not shown).

Mutations in wild type arise by two major pathways: Errors during replication and mutagenic bypass of premutagenic lesions in the DNA. Typical errors originating during replication are frameshifts and base substitution mutations. Premutagenic lesions have—depending on their chemical structure—a very diverse, but often lesion-specific mutagenic potential. O^6-methylguanine, for example, induces mainly G/C to A/T transitions (Eadie *et al.* 1984; Loechler *et al.* 1984) and the bypass of AP sites by Rev1 results in the insertion of a C opposite the AP site (Nelson *et al.* 1996a). The underrepresentation of frameshifts in the *mph1* mutant is in accord with the notion derived from the *REV3* dependence of the mutator phenotype that the error frequency during replication is not increased. Thus, the major source for the increased number of mutations in *mph1* mutants should be an increased number of premutagenic lesions that are bypassed by the error-prone pathway. If, however, the mutation spectrum is not slanted toward a particular type of mutation as is found for other repair mutants (Kunz *et al.* 1998), the simplest explanation is not that the distribution of the different types of premutagenic lesions is changed in *mph1* mutants but rather the frequency of all of them. This assumption is also in accordance with the observation that the mutator phenotype is not epistatic with any of the mutants tested. The frequency of premutagenic lesions might be changed either by an altered rate of formation, an altered removal rate, or by changes in both of them.

It seems unlikely that *MPH1* constitutes a new, independent repair pathway, which could correct all the various lesions. A more plausible explanation would be that *MPH1* is acting either upstream of repair, decreasing the rate of lesion formation, or as an accessory factor for the known repair pathways, increasing the removal rate. Such an accessory function could be modulation or reestablisment of chromatin, which has been shown to affect repair efficiency (Smerdon and Conconi 1999). Furthermore, mutants in subunits of the chromatin assembly factor I required for reassembly of chromatin after replication are UV sensitive (Kaufman *et al.* 1997; Game and Kaufman 1999) and it has been shown

that this factor also couples chromatin assembly to nucleotide excision repair in Xenopus (Gaillard *et al.* 1996). This would, however, not explain the apparent differences in processing of UV-induced damage *vs.* 4-NQO adducts, a phenotype also observed for Werner mutant cells (Fujiwara *et al.* 1977; Ogburn *et al.* 1997). This could be explained if Mph1 affects the rate of lesion formation, *e.g.*, by modulating the susceptibility of DNA to chemical attack. Mph1 may help to return DNA from a more vulnerable state, *e.g.*, after replication or transcription, to a more protected state again. At the present state, any guesses must be speculative. It might be the compaction of chromatin, the amount of unwound DNA in a cell, or changes in the secondary structure of DNA. To reconcile the lack of UV and X-ray sensitivity in *mph1* mutants with the increased sensitivity to MMS, EMS, and 4-NQO, the "vulnerable" state should affect the rate of lesion formation for the latter three treatments but to a lesser extent than that of the first two treatments. A variety of studies indicate that the rate of lesion formation is influenced differentially by chromatin structure depending on the DNA-damaging agents. Whereas, *e.g.*, the distribution of UV-induced cyclobutane pyrimidine dimers is largely unaffected by chromatin (Williams and Friedberg 1979; Niggli and Cerutti 1982; Mitchell *et al.* 1990), most chemically induced DNA adducts show a pronounced preference for linker DNA (Ramanathan *et al.* 1976; Metzger *et al.* 1977; Kuo and Hsu 1978; Jahn and Litman 1979; Sudhakar *et al.* 1979; Feldman *et al.* 1980; Kaneko and Cerutti 1980, 1982; Berkowitz and Silk 1981). Thus, changes in chromatin structure in the *mph1* mutant might be responsible for the different sensitivities.

At any rate, if mammals do possess *MPH1* orthologous genes as strongly suggested by the available EST clones, defects in this gene in humans may greatly influence the susceptibility of affected individuals to genotoxic chemicals and thus increase cancer risk as a result of exposure to these chemicals.

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