Interactions of Exo1p with components of MutLα in *Saccharomyces cerevisiae*

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Previously, we reported evidence suggesting that *Saccharomyces cerevisiae* MutLα, composed of Mlh1p and Pms1p, was a functional member of the gyrase b/Hsp90/MutL (GHL) dimeric ATPase superfamily characterized by highly conserved ATPase domains. Similar to other GHL ATPases, these putative ATPase domains of MutLα may be important for the recruitment and/or activation of downstream effectors. One downstream effector candidate is Exo1p, a 5′-3′ double stranded DNA exonuclease that has previously been implicated in DNA mismatch repair (MMR). Here we report yeast two-hybrid results suggesting that Exo1p can interact physically with MutLα through the Mlh1p subunit. We also report epistasis analysis involving MutLα ATPase mutations combined with *exo1*Δ. One interpretation of our genetic results is that MutLα ATPase domains function to direct Exo1p and other functionally redundant exonucleases during MMR. Finally, our results show that much of the increase in spontaneous mutation observed in an *exo1*Δ strain is *REV3*-dependent, in turn suggesting that Exo1p is also involved in one or more MMR-independent mutation avoidance pathways.

DNA mismatch repair (MMR) is a highly conserved genome fidelity process. Phenotypes of MMR deficiency are diverse, ranging from increased spontaneous mutation rates to cancer predisposition (1, 2). Mutation avoidance is a major function of MMR and can be dissected by using facile model systems such as *Escherichia coli* and the yeast *Saccharomyces cerevisiae* (2–5). For *E. coli* MMR, all essential genes have been identified, and their gene products have been used to reconstitute a MMR reaction *in vitro* (5). The three central components of this pathway are MutS, MutL, and MutH. A MutS dimer binds to a mispair, followed by ATP-dependent complex formation with a MutL dimer. The MutS/MutL/mispair ternary complex is thought to direct downstream events, including methylation-dependent nascent strand nicking by MutH, excision of the nascent strand, repair synthesis, and ligation. Recent studies suggest that the coordination of multiple downstream events, including nicking and excision, are facilitated by the ATPase activities of the MutL dimer (6–8).

MMR-mediated mutation avoidance in *S. cerevisiae* involves multiple MutS homologues (MSH) and MutL homologues (MLH) (2–4). For mutation avoidance, yeast use two partially redundant MutS-like activities, MutSα (Msh2p-Msh6p heterodimer) (9–16) and MutSβ (Msh2p-Msh3p heterodimer) (10, 11). Similarly, yeast use two MutL-like activities, MutLα (Mlh1p-Pms1p heterodimer) (17–20) and MutLβ (Mlh1p-Mlh3p heterodimer) (21, 22), although, based on genetic analysis, MutLα is the major MutL-like activity. Yeast have no known sequence or structural MutH homologue, partly exemplifying the lack of insight into the mechanism of strand discrimination.

In a previous study, we reported studies suggesting that *S. cerevisiae* MutLα is a member of the gyrase b/Hsp90/MutL (GHL) dimeric ATPase superfamily, which is characterized by highly conserved ATPase motifs (23). Although direct evidence for ATP-binding and hydrolysis activity has not been reported, our genetic and biochemical results suggest that, similar to other GHL ATPases, yeast MutLα undergoes ATP-dependent conformational changes, highlighted by dimerization of the NH_2-terminal ATPase domains (23, 24). These ATP-dependent conformational changes in MutLα and resultant NH_2-terminal dimerization between Mlh1p and Pms1p protomers appear to be critical for MMR because mutations affecting these activities compromise yeast MMR *in vivo* (23). Analogous to other GHL ATPases (7, 25–32), the apparent ATP-dependent conformational changes and the NH_2-terminal dimerization of MutLα may help to direct downstream effectors in the MMR process. One such downstream effector candidate is the 5′-3′ exonuclease Exo1p, originally identified as a mutator gene in *Schizosaccharomyces pombe* (33, 34), and later reported for *S. cerevisiae* as a "two-hybrid" interactor with Mlh1p (35) and Msh2p (36). Moreover, previous genetic studies showed essentially identical phenotypic effects between *exo1*Δ and a missense mutation in a residue predicted to be critical for exonuclease activity but not for structural integrity of the protein. These findings were consistent with Exo1p performing a catalytic role during MutSα-dependent MMR rather than being limited to a structural role (37).

Here, we report results suggesting both physical and genetic interactions between *EXO1* and the components of MutLα, Mlh1p and Pms1p. Specifically, we characterized further our initial two-hybrid interaction between Mlh1p and Exo1p. More interestingly, we report genetic interactions between mutations in *MLH1*, *PMS1*, and *EXO1* that suggest that one function of the MutLα ATPase domains is to direct Exo1p and possibly other exonucleases during MMR-mediated mutation avoidance. Finally, the results suggest that Exo1p is also involved in one or more MMR-independent mutation avoidance pathways.

Materials and Methods

Strains and Media. *E. coli* strain DH-10B was used for plasmid construction and amplification. Bacterial and yeast strains were grown under conditions described (17). Yeast transformations were performed by the polyethylene glycol/lithium acetate method (38).

Disruptions of *EXO1* were generated as described previously (36). An *exo1*::*HIS3* disruption cassette was generated by PCR using strain EAY618 (E. Alani, Cornell Univ., Ithaca, NY), and transformed into yeast.

Disruptions of *REV3* were created by transforming the pYPG101 construct (D. Hinkle, Rochester University, Rochester, NY) after *Kpn*I digestion and selecting for Ura^+ prototrophs. Genotyping of strains was performed by PCR or Southern blot analysis (specifics are available on request). The generation of the other strains used in this study has been described (23).

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Abbreviations: MMR, mismatch repair; MutLα, Mlh1p and Pms1p; MSH, MutS homologues; MLH, MutL homologues; FS, frameshift; BS, base substitution; CI, confidence intervals; GHL, gyrase b/Hsp90/MutL.

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Plasmid Construction. All DNA manipulations were performed by using standard molecular biology procedures (39). All automated sequencing was done with an Applied Biosystems automated sequencer.

pBTM-MSH2 and pBTM-MSH6 were constructed by cloning the coding sequences for yeast *MSH2* and *MSH6* into the two-hybrid "bait" vector pBTM116. The other constructs used in this study were described (17).

Two-Hybrid Screening and Mating and β-Galactosidase Assays. The two-hybrid screening was performed as described (40) with a yeast cDNA expression library (S. Elledge, Baylor College of Medicine, Waco, TX). Candidates were retested directly "one-on-one" by mating as described (23). Growth at 30°C for 2–3 days on −uracil −tryptophan −leucine (−UTL) plates indicated efficiency of mating, whereas growth on −tryptophan −histidine −uracil −leucine −lysine (−THULL) plates indicated "bait–prey" interaction.

Diploid L40/AMR70 is homozygous for a second chromosomal lexA-GAL4A reporter system, *URA3*::(*lexAop*) $_8$-*lacZ*. β-Galactosidase liquid assays were performed as described (17). One β-galactosidase unit = [$(OD_{420}/OD_{600})60$]/ min, where OD_{420} and OD_{600} are the optical densities at 420 and 600 nm, respectively.

Measurement of Mutation Rates and *CAN1* Mutational Spectra Analysis. Strains were streak purified, individual colonies were grown to saturation in YPD medium, then various dilutions were plated onto complete synthetic medium, −threonine, and +canavanine (+CAN) [at 60 μg/ml] plates, and colonies were counted after 2–3 days growth at 30°C. Rates were determined as described (17). The 95% confidence intervals (CI) of relative mutation rates were determined by using PRISM 2.0a software (GraphPad, San Diego).

Canavanine-resistance (*CAN*^{R}) mutations were identified from genomic preparations by using the glass bead lysis method, followed by PCR of the *CAN1* gene as described (23), and direct sequencing of the QIAquick (Qiagen, Chatsworth, CA) purified PCR amplicon. χ^2 analysis was used to determine whether changes in mutational spectra were statistically significant ($P <$ 0.05). Rates of frameshifts (FS) and base substitutions (BS) at *CAN1* were calculated by using absolute mutation rates determined at *CAN1* multiplied by the frequency with which FS or BS mutations occurred in the particular strain. As the calculated rates of FS and BS at *CAN1* in Table 3 possess the product of two different forms of error, we were unable to perform statistical analysis on these values.

Results

Experimental Rationale. In a previous report we referred for convenience to the mutations *mlh1-E31A* and *pms1-E61A*, which are predicted to affect ATP hydrolysis with little or no effect on ATP binding, as "ATP-hydrolysis" mutations (23). Similarly, we referred to a second pair of mutations, *mlh1-G98A* and *pms1-G128A*, which are predicted to cause a deficiency in ATP-binding and/or ATP-binding-dependent conformational changes, as "ATP-binding" mutations. Interestingly, we found that alterations in both of these putative ATPase motifs of Mlh1p produced more severe effects on mutation avoidance than did the corresponding "ATPase" mutations in Pms1p. These differential effects of the *mlh1* ATPase mutations versus the *pms1* ATPase mutations were not because of partially redundant functions of *MLH3* and *PMS1*, and therefore suggested a functional asymmetry within the MutLα heterodimer (23).

As discussed above, one possible function of the ATPase domains of MutLα is the recruitment and/or activation of additional proteins. One candidate is Exo1p, which we identified previously from a two-hybrid screen as an Mlh1p interactor (35)

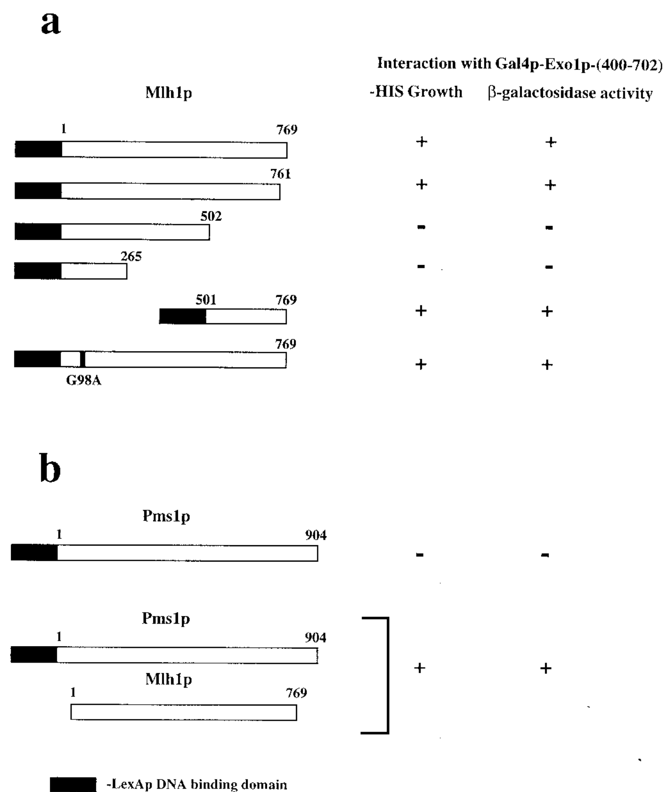

Fig. 1. Exo1p and MMR proteins interact in a yeast two-hybrid assay. (*a*) Boxes correspond to Mlh1p "bait" constructs tested for interaction. The residues of Mlh1p included in the fusions are indicated above each respective construct. The amino acid substitution G98A made in Mlh1p is designated by a black bar within the construct box. Interaction is scored as growth on −histidine media and >0.5 β-galactosidase units with the substrate *o*-nitrophenylβ-D-galactosidase (ONPG) as described in *Materials and Methods*. (*b*) Full-length LexAp-Pms1p fusion alone or in a "three-hybrid" assay with native Mlh1p coexpressed was tested for interaction with the Gal4p-Exo1p-(400–702) fusion using the same analysis as in *a*.

(see below). In the present study we have analyzed further initial two-hybrid results and have performed epistasis analysis with the MutLα "ATPase" mutations combined with *exo1*Δ. The epistasis analyses included both mutation-rate measurements at *hom3-10* and *CAN1*, and mutational spectrum analysis using the *CAN1* reporter. The *hom3-10* allele enables a reversion assay that reports single T·A base pair deletions in a run of 7 T·A base pairs, and has been considered diagnostic for defects in MMR (41). In contrast, forward mutation at *CAN1* shows a wide variety of inactivating mutations, the spectra of which can be determined by DNA sequencing (41). Finally, because *MLH3* is partially redundant with *PMS1* for mutation avoidance (21–23), we examined the mutational spectra of relevant *pms1* ATPase mutant strains in an *mlh3*Δ background.

Exo1p Interacts with Mlh1p by Yeast Two-Hybrid Assay. Using full-length Mlh1p as a bait in a two-hybrid screen, we recovered a COOH-terminal fragment of Exo1p (residues 400–702). This Exo1p fragment was retested directly, and was shown to interact with LexAp-Mlh1p as depicted in Fig. 1*a*. Using deletion constructs, we mapped the minimal region of Mlh1p required to interact with this COOH-terminal fragment of Exo1p to residues 501–761 of Mlh1p (Fig. 1*a*). To address the question whether ATP-binding or ATP-binding-dependent conformational changes by Mlh1p were necessary for interaction with Exo1p, we examined LexAp-mlh1-G98A for interaction with Exo1p. As

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Table 1. Mutation rates of *exo1Δ* strains in yeast MutL mutant backgrounds

Strain	Relevant genotype	Fold mutator rate (95% CI)*	
		hom3-10†	*CAN*‡
GCY35§	Wild type	1 (0–2.3)	1 (0.1–2)
PTY100	*mlh1Δ*	1,118 (858–1378)	32 (22–41)
PTY101§	*pms1Δ*	1,212 (1017–1408)	28 (15–41)
PTY105	*exo1Δ*	8 (0–24)	9 (5–14)
PTY106	*mlh1Δexo1Δ*	1,227 (134–2320)	37 (30–43)
PTY107	*pms1Δexo1Δ*	1,097 (253–1940)	35 (11–60)
PTY200	*mlh1-E31A*	316 (170–461)	9 (3–14)
PTY207	*mlh1-E31A exo1Δ*	1,219 (779–1660)	46 (21–72)
PTY201§¶	*pms1-E61A*	19 (6–32)	1 (0.5–2)
PTY204¶	*pms1-E61A exo1Δ*	517 (163–872)	15 (9–20)
PTY300§	*mlh1-G98A*	725 (524–926)	22 (10–34)
PTY307	*mlh1-G98A exo1Δ*	922 (361–1482)	36 (22–50)
PTY301§¶	*pms1-G128A*	78 (33–122)	4 (3–5)
PTY304¶	*pms1-G128A exo1Δ*	611 (361–862)	24 (20–27)

*From two to six determinations with 5–11 cultures per experiment.
†Relative to wild-type GCY35 rate of 9.9×10^{-9}.
‡Relative to wild-type GCY35 rate of 3.01×10^{-7}.
§These rates are taken from Tran and Liskay (23).
¶Codeletion of *MLH3* did not change the rates significantly (two-tailed Mann–Whitney test, $P > 0.05$).

shown in Fig. 1*a*, this Mlh1p mutant, which is predicted to have compromised ATP-binding activity, retained ability to interact with Gal4p-Exo1p-(400–702).

We also tested the Exo1p clone against a panel of other MMR proteins. This COOH-terminal fragment of Exo1p interacted with full-length LexAp-Msh2p (data not shown) as in a previous report (36), but not with full-length LexAp-Msh6p (data not shown) or LexAp-Pms1p fusions (Fig. 1*b*). However, we found that the COOH-terminal fragment of Exo1p did interact with LexAp-Pms1p in a "three-hybrid" assay in which native Mlh1p was coexpressed (Fig. 1*b*). Taken together, these results suggest that the COOH-terminal fragment of Exo1p interacts with the Mlh1p subunit of MutLα.

Epistasis Analysis of *hom3–10* Reversion Rates for *mlh1* and *pms1* "ATPase" Mutations and *exo1Δ*. To detect genetic interactions between components of MutLα and *EXO1*, we determined mutation rates in a series of single and double mutants (Table 1). The most striking result was that either the *pms1-E61A* or the *pms1-G128A* mutation, when combined with *exo1Δ*, produced greater-than-additive mutation rates (with 95% CI) by using *hom3–10* (Table 1; PTY204 > PTY105 + PTY201, and PTY304 > PTY105 + PTY301). Likewise, using *hom3–10* reversion, the *mlh1-E31A* mutation appeared to synergize for spontaneous mutation in combination with *exo1Δ* (Table 1; PTY207 > PTY105 + PTY200). In contrast, the small effect of *exo1Δ* at *hom3–10* relative to the large effect produced by the *mlh1Δ*, *pms1Δ*, or *mlh1-G98A* mutations prevented us from making conclusions regarding epistasis with the corresponding double mutants. Using 95% CIs at *CAN1*, we observed a synergistic interaction for mutation rates only for the *pms1-G128A* and *exo1Δ* mutations (Table 1). However, as presented below, determination of *CAN1* mutational spectra suggested that similar to findings at *hom3–10*, *exo1Δ* interacted with *mlh1-E31A*, *pms1-E61A*, or *pms1-G128A* in a greater-than-additive fashion for FS mutations in short mononucleotide runs.

Analysis of *CAN1* Mutation. To elucidate further genetic interactions between *MLH1*, *PMS1*, and *EXO1*, we determined the *CAN1* mutational spectra for a subset of single and double mutant strains. As shown in Table 2, all single *mlh1* and *pms1* mutant strains examined showed a *CAN1* spectrum characterized by a FS to BS mutation ratio (FS/BS) of 2 or greater. A 2- to 3-fold preponderance of FS over BS mutations has been shown previously for *msh2Δ* strains (10). Based on other studies (10, 42) and our findings with *mlh1* and *pms1* null strains, we will consider an excess of FS over BS mutations as reflective of a defect in MMR. In contrast, the *exo1Δ* strain exhibited a spectrum that was different from a MMR-defective strain, e.g.,

Table 2. Summary of mutational spectra at *CAN1*

Strain	Relevant genotype	Class of mutation				FS/BS†
		FS		BS	Complex	
		Frequency (%)	Type*	Frequency (%)	Frequency (%)	
GCY35‡§	Wild type	7/20 (35)	86:14	11/20 (55)	2/20 (10)¶	0.6
PTY100‡	*mlh1Δ*	8/10 (80)	100:0	2/10 (20)	0/10 (0)	4.0
PTY200‡	*mlh1-E31A*	12/20 (60)	75:25	8/20 (40)	0/20 (0)	1.9‖
PTY300‡	*mlh1-G98A*	17/20 (85)	99:12	3/20 (15)	0/20 (0)	5.7
PTY101‡	*pms1Δ*	8/10 (80)	62:38	2/10 (20)	0/10 (0)	4.0
PTY104‡	*pms1Δmlh3Δ*	8/10 (80)	62:38	2/10 (20)	0/10 (0)	4.0
PTY302‡	*pms1-G128A mlh3Δ*	18/25 (72)	94:6	7/25 (28)	0/25 (0)	3.3‖
PTY105	*exo1Δ*	9/20 (45)	67:33	11/20 (55)	0/20 (0)	0.8
PTY106	*mlh1Δexo1Δ*	13/19 (68)	100:0	6/19 (32)	0/19 (0)	2.2
PTY207	*mlh1-E31A exo1Δ*	15/19 (79)	87:13	4/19 (21)	0/19 (0)	3.8
PTY307	*mlh1-G98A exo1Δ*	9/20 (45)	89:11	11/20 (55)	0/20 (0)	0.8
PTY107	*pms1Δexo1Δ*	12/18 (67)	92:8	6/18 (33)	0/18 (0)	2.0
PTY205	*pms1-E61A exo1Δmlh3Δ*	15/20 (75)	100:0	5/20 (25)	0/20 (0)	3.0
PTY305	*pms1-G128A exo1Δ mlh3Δ*	13/20 (65)	100:0	7/20 (35)	0/20 (0)	1.9

*Ratio of contractions:expansions.
†FS:BS ratio.
‡Spectrum taken from Tran and Liskay (23).
§The *mlh3Δ* strain is no different from wild type (R. D. Kolodner, personal communication) and *pms1-E31A mlh3Δ* is not a mutator at *CAN1* (Table 1).
¶Duplication events with direct repeats.
‖This value is the FS/BS ratio with the wild-type spectrum contribution subtracted.

Table 3. Relative estimated rates of FS and BS mutations at *CAN1*

Strain	Relevant genotype	Fold mutator rate	
		FS*	BS†
GCY35	Wild type	1	1
PTY100	*mlh1Δ*	73	12
PTY200	*mlh1-E31A*	15	6
PTY300	*mlh1-G98A*	53	6
PTY101	*pms1Δ*	64	10
PTY104	*pms1Δmlh3Δ*	63	10
PTY202	*pms1-E61A mlh3Δ*	1‡	1‡
PTY302	*pms1-G128A mlh3Δ*	9	2
PTY105	*exo1Δ*	12	9
PTY106	*mlh1Δexo1Δ*	72	22
PTY207	*mlh1-E31A exo1Δ*	104	18
PTY307	*mlh1-G98A exo1Δ*	46	36
PTY107	*pms1Δexo1Δ*	68	21
PTY205	*pms1-E61A exo1Δmlh3Δ*	31	6
PTY305	*pms1-G128A exo1Δmlh3Δ*	38	13

Relative rates were calculated from Table 1 and frequency of FS and BS mutations from Table 2 as described in *Materials and Methods.*
*Relative to the wild-type rate of 1.05×10^{-7} for FS at *CAN1.*
†Relative to the wild-type rate of 1.66×10^{-7} for BS at *CAN1.*
‡Assumed that PTY202 mimics the wild type for ratio of FS/BS.

mlh1Δ ($P < 0.001$), but not different from the wild-type strain ($P > 0.1$) (Table 2). Although no single pairwise comparison was statistically significant ($P > 0.1$), deletion of *EXO1* combined with any of the *mlh1* or *pms1* single mutations produced, as a general trend, an apparent shift in spectrum toward more BS mutations (Table 2). Therefore, initial analysis of the *CAN1* mutations suggested that *exo1* deficiency alone did not produce a spectrum characteristic of MMR deficiency, and that combining *exo1Δ* with several of the *pms1* or *mlh1* mutations appeared to cause a shift in the FS/BS ratio toward one more characteristic of wild-type (or *exo1Δ*) cells.

Another way to analyze mutation spectrum data is to estimate the rates of specific types of mutations arising at the mutation-reporter locus (21, 43, 44). Using the *CAN1* mutation rates (from Table 1) and the frequency of FS and BS mutations arising at *CAN1* (from Table 2), we estimated the rates of FS and BS mutations at *CAN1* for the various single and double mutant strains. We present these rates relative to wild type in Table 3. Because of the limited sample size of *can1* mutants analyzed for each strain and because we were unable to perform statistical analysis on the values shown in Table 3 (see *Materials And Methods*), we could not make strong arguments regarding epistasis, additivity, or synergy. Despite this limitation, we observed two interesting trends. (*i*) Similar to the *hom3–10* data described above, combination of the *exo1Δ* mutation with the *pms1-E61A*, *pms1-G128A*, or *mlh1-E31A* mutation appeared to result in greater than additive effects on FS mutation rates at *CAN1*. (*ii*) For *mlh1Δ* and *exo1Δ*, *mlh1-E31A* and *exo1Δ*, and *pms1Δ* and *exo1Δ* mutant combinations, there appeared to be a general trend of additivity for BS mutation rates at *CAN1*. Taken together, there appeared to be synergistic interactions between the *mlh1-E31A* and *pms1* "ATPase" mutations and *exo1Δ* for FS mutation rates, which we interpret as indicative of MMR deficiency. One idea that is consistent with our observations is that the ATPase domains of MutLα are important for directing Exo1p and factors functionally redundant with Exo1p during MMR. In addition, the trend of apparent additivity for BS mutation rates suggested that BS mutations are being contributed by more than one pathway that act in parallel to one another.

Table 4. Effect of *rev3Δ* on mutation rates

Strain	Relevant genotype	Fold mutator rate (95% CI)*	
		hom3–10†	*CAN*R‡
GCY35§	Wild type	1 (0–2.3)	1 (0.1–2)
PTY100§	*mlh1Δ*	1,118 (858–1378)	32 (22–41)
PTY105§	*exo1Δ*	8 (0–24)	9 (5–14)
PTY110	*rev3Δ*	0.3 (0.1–0.5)	0.7 (0–2)
PTY111	*rev3Δexo1Δ*	2 (0–4)	1 (0.6–3)
PTY112	*rev3Δmlh1Δ*	911 (156–1668)	28 (3–53)
PTY204§	*pms1-E61A exo1Δ*	517 (163–872)	15 (9–20)
PTY210	*pms1-E61A exo1Δ rev3Δ*	197 (121–273)	13 (10–15)
PTY304§	*pms1-G128A exo1Δ*	611 (361–862)	24 (20–27)
PTY310	*pms1-G128A exo1Δrev3Δ*	281 (192–370)	19 (12–25)

*Experiments repeated two to four times with 5–11 cultures per experiment.
†Relative to wild-type GCY35 rate of 9.9×10^{-9}.
‡Relative to wild-type GCY35 rate of 3.01×10^{-7}.
§Rates taken from Table 1.

The Majority of Spontaneous Mutation at *CAN1* in an *exo1Δ* Strain Is *REV3*-Dependent. The mutational spectrum at *CAN1* observed for the *exo1Δ* single mutant and the general trend of additive interactions between MutLα mutations and *exo1Δ* for BS rates at *CAN1* suggested that *EXO1* also may be involved in MMR-independent pathways for mutation avoidance. Because *rev3Δ* strains are slightly hypomutable spontaneously (45), and because *rev3Δ* can suppress the mutator phenotype of strains in which certain mutation avoidance pathways are defective (46–50), we examined the effect of *rev3Δ* on the *exo1Δ* mutator phenotype. Interestingly, although the *CAN1* mutation rate of the *exo1Δ* strain (PTY105) was ≈9-fold higher than wild type (GCY35), the rate of the double *rev3Δ exo1Δ* strain (PTY111) was not statistically different from the wild-type rate (Table 4). These results suggested that the *exo1Δ* mutator phenotype at *CAN1* was largely *REV3*-dependent.

As described above, we observed either significant (for *hom3–10*) or apparent (for *CAN1*) synergism between the *pms1-E61A* and *pms1-G128A* mutations and *exo1Δ* for FS mutation rates (Tables 1 and 3). To determine whether this pattern of FS synergy observed between *pms1-E61A* and *pms1-G128A* mutations and *exo1Δ* depended on *REV3* function, we analyzed the effect of *rev3Δ* mutation on the *pms1-E61A exo1Δ* and *pms1-G128A exo1Δ* double mutant strains (Table 4). Notably, we observed that the mutation rate at either *hom3–10* or *CAN1* of the double mutants was not reduced significantly by *rev3Δ* (Table 4). Importantly, we determined that *mlh1Δ rev3Δ* and *msh2Δ rev3Δ* strains had mutation rates essentially the same as *mlh1Δ* or *msh2Δ* strains, respectively, suggesting that the majority of MMR-dependent mutations are *not REV3*-dependent (Table 4 and data not shown). These results are consistent with the hypothesis that the synergy seen between the *pms1* "ATPase" mutations and *exo1Δ* is reflective of a defect in MMR. Taken together, the results suggest that *EXO1* can be involved in at least two mutation avoidance pathways, an Mlh1p/Pms1p-dependent MMR pathway and a MMR-independent but *REV3*-dependent pathway.

Discussion

In this study, we report evidence for physical and genetic interactions between *S. cerevisiae* Exo1p and the components of MutLα, Mlh1p and Pms1p. In brief, using yeast two-hybrid and three-hybrid analyses, we observed that a COOH-terminal fragment of Exo1p interacted with MutLα through a COOH-

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fragment of Mlh1p. Epistasis analyses revealed greater than additive effects for spontaneous FS mutation rates, characteristic of a MMR defect, for strains containing the *mlh1-E31A*, *pms1-E61A*, or *pms1-G128A* mutations, located in the putative ATPase domains of MutLα, and combined with *exo1*Δ mutation. In addition, and in contrast to our findings for either *mlh1*Δ or *pms1*Δ strains, the *CAN1* mutational spectrum of an *exo1*Δ strain was not consistent with a defect in MMR. In agreement with these *CAN1* spectra comparisons, we found that, in contrast to the *mlh1*Δ or *msh2*Δ mutator phenotypes, much of the mutator effect at *CAN1* in an *exo1*Δ strain was, in fact, *REV3* dependent.

A previous report demonstrated physical interaction between Exo1p and *MSH2* (36). Here, using two-hybrid analysis, we show that a COOH-terminal domain of Mlh1p required for interaction with Pms1p (17, 52), Mlh2p, and Mlh3p (52) also interacted with a COOH-terminal fragment of Exo1p (Fig. 1*a*). Furthermore, using a three-hybrid scheme, we provide evidence that Exo1p interacts with MutLα through the COOH-terminal of Mlh1p (Fig. 1*b*). Similar to studies with yeast and human Exo1p showing interaction with yeast and human Msh2p (36, 51, 53), respectively, we identified interactions between a COOH-terminal fragment of Exo1p and Mlh1p. These findings raise the possibility that, in eukaryotes, a conserved COOH-terminal domain of Exo1p is responsible for interactions with several classes of MMR proteins. Whether these interactions can occur concomitantly or only independently is of interest and will require further study. In addition, we found that a mutant form of Mlh1p, predicted to have reduced ATP binding, retained ability to interact with Exo1p, suggesting that ATP-dependent conformational changes in Mlh1p may not be necessary for interaction with Exo1p. Findings with similar mutant forms of *E. coli* MutL suggested that the ATPase activity of MutL was not necessary for interaction with MutH and UvrD, but was required for activation of these components during incision and excision (7). The genetic analysis (see below) addresses the issue of whether the ATPase domains of MutLα might be required for activation of downstream candidates in MMR, such as Exo1p.

We performed epistasis analysis using MutLα "ATPase" mutations and *exo1*Δ because of the evidence for physical interaction discussed above and our previous studies, which suggested that MutLα undergoes ATP-dependent NH_2-terminal conformational changes (23, 24) that in turn may be important for coordinating downstream events. Using two mutation reporters, we observed genetic interactions for mutation avoidance between *mlh1* or *pms1* mutations and *exo1*Δ. Of primary interest, the two *pms1* "ATPase" mutations and *mlh1-E31A* mutation each synergized with *exo1*Δ for FS mutation rates. Similar synergistic interactions for mutation avoidance between weak mutator alleles of *mlh1* or *pms1* and *exo1*Δ were identified by the Kolodner group in a genetic screen designed to identify second-site mutations that would synthetically enhance the weak mutator phenotype of an *exo1*Δ strain (73). One explanation for the synergy is that the Mlh1p ATP-hydrolysis motif, for example, is necessary to coordinate a factor(s) [e.g., another exonuclease(s)] that is functionally redundant with Exo1p. Similarly, the synergy observed between each of the two *pms1* "ATPase" mutations and *exo1*Δ suggests that these Pms1p motifs are also important for coordinating a factor(s) redundant with Exo1p. In other words, as one hypothesis, we suggest that these specific MutLα ATPase motif mutations mimic defects in factors redundant to Exo1p, at least in terms of MMR function, such that the combination of these MutLα mutations and *exo1*Δ (e.g., *pms1-E61A* and *exo1*Δ) mimics inactivation of *EXO1* and the putative "redundant" gene(s). This explanation is consistent with the idea that the ATPase activity of *E. coli* MutL helps to coordinate both incision (6–8) and excision (7).

The existence of redundant factors for Exo1p agrees with the lack of a strong mutator phenotype for *exo1*Δ strains (36, 54) and *in vitro* evidence for bidirectional repair capability for eukaryotic MMR (55). In light of the proposal that MMR and the replication machinery may interact directly (40, 56, 57), an alternative explanation is that the MutLα ATPase motif mutations may impinge on replication fidelity and, in conjunction with *exo1*Δ, result in synergistic increases in mutation similar to those proposed for DNA polymerase proofreading defects combined with *exo1*Δ (54). Another explanation for a synergistic interaction for FS mutation rates is that *exo1*Δ combined with specific MutLα ATPase mutations may result in the "structural collapse" of a complex required for MMR. We do not favor exclusively such a structural requirement for Exo1p during MMR because studies using an exonuclease-deficient *exo1* allele (37) that appears to be structurally intact (P.T.T. and R.M.L., unpublished data) also resulted in a synergistic increase in FS mutation rates when combined with either of the *pms1* ATPase mutations (P.T.T. and R.M.L., unpublished data). Although a structural role for Exo1p in MMR is possible based on the studies of others (73), we favor the idea put forth previously (37) that Exo1p can have a catalytic role in MMR.

As discussed above, the *CAN1* mutational spectrum for the *exo1*Δ strain was not characteristic of known MMR-defective strains, e.g., *mlh1*Δ. In addition, when we estimated rates of FS and BS mutations at *CAN1* in various single and double mutants we observed two general trends. (*i*) Similar to the *hom3–10* results, the *mlh1*-E31A mutation and both the *pms1* ATPase mutations each appeared to synergize with *exo1*Δ for FS mutation rates at *CAN1*. (*ii*) Using a limited number of BS events at *CAN1*, estimated rates of BS mutations in the single and double mutant suggested an additive effect when *exo1*Δ was combined with any of several different MutLα mutations. Together, the results suggest involvement of *EXO1* in a MutLα-dependent MMR pathway, based on FS mutation rates, and a MMR-independent mutation avoidance pathway, based on BS mutations rates.

What is the nature of the *EXO1*-dependent, MMR-independent pathway for mutation avoidance? Recent studies have shown that the mutator phenotypes observed in strains defective in several DNA repair pathways are largely dependent on the *REV3* gene (46–50). One explanation for these findings is that when certain DNA repair pathways are blocked, spontaneously occurring DNA lesions are "funneled" into the REV3-dependent error-prone replication bypass pathway (48). Therefore, we characterized the effect of *rev3*Δ on the *exo1*Δ mutator phenotype. Interestingly, we found that *CAN1* mutation rates in the *exo1*Δ *rev3*Δ strain were reduced to near wild-type, indicating that most of the *CAN1* mutator phenotype of *exo1*Δ was dependent on *REV3*. Rev3p functions as a component of the error-prone polymerase ζ (Rev3p-Rev7p) to bypass DNA lesions that stall the replicative polymerases (45). Our data would therefore suggest that Exo1p assists in an error-free process acting on spontaneous DNA lesions. Because Exo1p has been implicated in several DNA metabolic pathways, such as repair of UV damage, recombination, and replication (58–64), further studies are required to clarify the proposed relationship between *REV3* and *EXO1*. In contrast, *rev3*Δ did not significantly reduce the rate of mutation in either an *mlh1*Δ or *msh2*Δ strain, suggesting, not surprisingly, that the mutator phenotype of a MMR-defective strain is not *REV3*-dependent. Importantly, the synergistic interaction seen between *exo1*Δ and either of the two *pms1* "ATPase" mutations was *not REV3*-dependent, consistent with our hypothesis that the synergy observed for FS mutation rates reflected a defect in MMR. As a whole, the results suggest that *EXO1* can be involved in MMR-dependent and MMR-independent mutation avoidance pathways.

Until recently, mechanistic details of how the eukaryotic MutL homologues couple the mismatch binding activities of MutS homologues to downstream effectors in eukaryotes have

been scarce. Studies identifying MutL homologues as members of an emerging ATPase superfamily have provided a framework with which to examine MutL homologue function during MMR-dependent mutation avoidance (6, 23, 29, 65). Based on the results presented here, we suggest that one function for the MutLα ATPase domains in *S. cerevisiae* is to coordinate Exo1p and redundant activities during mutation avoidance. Because MMR proteins also have been shown to function in other pathways, such as meiotic (52, 66–68) and homeologous recombination (69–72), the use of MutLα ATPase mutations may be important for a more complete understanding of the role of MMR proteins in other DNA transactions.

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