

Research Article

The *pol3-t* Hyperrecombination Phenotype and DNA Damage-Induced Recombination in *Saccharomyces cerevisiae* Is *RAD50* Dependent

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The DNA polymerase δ (*POL3/CDC2*) allele *pol3-t* of *Saccharomyces cerevisiae* has previously been shown to be sensitive to methylmethanesulfonate (MMS) and has been proposed to be involved in base excision repair. Our results, however, show that the *pol3-t* mutation is synergistic for MMS sensitivity with *MAG1*, a known base excision repair gene, but it is epistatic with *rad50 Δ* , suggesting that *POL3* may be involved not only in base excision repair but also in a *RAD50* dependent function. We further studied the interaction of *pol3-t* with *rad50 Δ* by examining their effect on spontaneous, MMS-, UV-, and ionizing radiation-induced intrachromosomal recombination. We found that *rad50 Δ* completely abolishes the elevated spontaneous frequency of intrachromosomal recombination in the *pol3-t* mutant and significantly decreases UV- and MMS-induced recombination in both *POL3* and *pol3-t* strains. Interestingly, *rad50 Δ* had no effect on γ -ray-induced recombination in both backgrounds between 0 and 50 Gy. Finally, the deletion of *RAD50* had no effect on the elevated frequency of homologous integration conferred by the *pol3-t* mutation. *RAD50* is possibly involved in resolution of replication forks that are stalled by mutagen-induced external DNA damage, or internal DNA damage produced by growing the *pol3-t* mutant at the restrictive temperature.

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1. Introduction

The *POL3/CDC2* gene of *Saccharomyces cerevisiae* encodes the catalytic subunit of the DNA polymerase δ . The coding sequence includes a catalytic domain, a nucleotide binding domain, and an exonuclease proofreading site [1]. Pol δ together with Pol α and Pol ϵ performs essential functions required for DNA replication. Pol α has a primase activity and is involved in initiation of both the leading and lagging strands [2]. Both Pol δ and Pol ϵ can extend the primers formed by Pol α [3, 4] and are proposed to be involved in nucleotide excision repair [5] and base excision repair [6, 7]. In addition, the DNA polymerase δ exonuclease is involved in postreplication repair [8, 9]. Several mutations of *POL3* have been characterized. Yeast strains lacking the proofreading exonuclease activity of the polymerase have a strong mutator phenotype [1]. The *pol3-t* mutation is located near the

catalytic domain outside the exonuclease domain in a region probably involved in nucleotide binding [1]. The *pol3-t* mutant allele, initially isolated as *tex1* mutant because it increased the rate of excision of a bacterial transposon within the yeast *LYS2* gene, also enhances intrachromosomal deletion recombination between short repeats of several base pairs separated by long inverted repeats [10]. The molecular analysis of the transposon excision events indicates that DNA replication slippage is most likely responsible for these excision events [11, 12]. Furthermore, the frequency of deletions between distant short repeats within *LYS2* or the *CAN1* gene is also increased many fold [11]. Finally, it has been shown that the same mutator phenotype as observed in the *pol3-t* mutation exists after repression of the *POL3* gene, indicating that the mutator phenotype may be due to low levels of *POL3* rather than to faulty effects of the *POL3* mutant proteins [1].

TABLE 1: *Saccharomyces cerevisiae* strains.

Name	Parent strain	Genotype	Source
RSY6		<i>MATa ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3 Δ5' -pRS6-his3 Δ3'</i>	[13]
YR50-1	RSY6	<i>MATa ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5' -pRS6-his3Δ3', rad50::hisG</i>	Schiestl collection
AGY30	RSY6	<i>MATa ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5' -pRS6-his3Δ3' pol3-t</i>	[14]
YMG1	RSY6	<i>MATa ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5' -pRS6-his3Δ3', MAG1::hisG</i>	Schiestl collection
AGY40	YMG1	<i>MATa ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5' -pRS6-his3Δ3', MAG1::hisG, pol3-t</i>	This study
AGY34	YR50-1	<i>MATa ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5' -pRS6-his3Δ3' pol3-t, rad50::hisG</i>	This study
RSY12		<i>MATa leu2-3,112 his3-11,15 URA3::HIS3</i>	[15]
YR50-12	RSY12	<i>MATa leu2-3,112 his3-11,15 URA3::HIS3, rad50::LEU2</i>	[16]
AGY38	RSY12	<i>MATa leu2-3,112 his3-11,15 URA3::HIS3, pol3-t</i>	[17]
AGY39	YR50-12	<i>MATa leu2-3,112 his3-11,15 URA3::HIS3-pol3-t, rad50::LEU2</i>	This study

RAD50 is involved in DNA double strand break repair by nonhomologous end joining and homologous recombination such as sister chromatid recombination and double strand break (DSB) processing. RAD50 together with XRS2 and MRE11 is part of the MRX complex which localizes to DSBs [18, 19]. Whereas wild type cells are much more radiation-sensitive in G1 (1.5% survival at 150 Gy) compared to G2 (70% survival), *mre11* mutant cells show about the same survival rate in both phases (0.6% versus 1%), indicating preferential MRX-mediated repair when sister chromatids are present in G2 [20]. In addition, radiation-induced sister chromatid recombination is reduced in the *mre11/rad50/xrs2* mutants [20]. The repair of stalled replication forks may involve recombination [21, 22]. The gene products 46/47 of the bacteriophage T4, homologs of Mre11/Rad50, are required for recombination-induced replication [23–25]. Recombination-induced replication also may be involved in DSB repair during G2 by synthesis dependent strand annealing [26], possibly explaining the MRX-mediated preferential repair in G2 cells [20].

Several mutants with elevated spontaneous intrachromosomal recombination frequencies have been isolated in *Saccharomyces cerevisiae* [27, 28]. Among them, a mutant allele of *CDC2/POL3*, which encodes the catalytic subunit of the DNA polymerase δ , increases deletion events [27]. Intrachromosomal deletion events between duplicated sequences may occur by several mechanisms such as intrachromatid exchange, single-strand annealing, one-sided invasion, unequal sister chromatid exchange or, sister chromatid conversion [13, 29–31]. We have previously shown that the *pol3-t* allele increases such intrachromosomal recombination events [14]. This hyperrecombination phenotype is partially dependent of *RAD1* and *RAD52* because the *pol3-t* mutation still enhances intrachromosomal recombination in the *rad1rad52* double mutant [14]. This suggests that

the hyperrecombination phenotype may depend on DNA genes other than *RAD52* or *RAD1*. Here, we report a further characterization of the *pol3-t* mutant. We investigated the effect of the *RAD50* gene involved in DSB repair [18], on the *pol3-t* phenotype by measuring methyl methane-sulfonate (MMS) sensitivity, the spontaneous as well as MMS-, UV-, and γ -ray-induced intrachromosomal recombination and, finally, the effect on homologous integration.

2. Materials and Methods

2.1. Media, Genetic, and Molecular Techniques. Complete media (YPAD), synthetic complete (SC), and drop-out (SD) media were prepared according to standard procedures. Magic Column (Promega, Madison, WI) was used for preparation of small-scale DNA. Yeast transformation was performed using the procedure described in 1995 [32].

2.2. Yeast Strains. The names and the genotypes of the strains used are listed in Table 1. Because *pol3-t* confers a temperature sensitive phenotype, all *pol3-t* strains were grown at 25°C [10]. Strains AGY34, AGY38, AGY39, and AGY40 were constructed by introducing the *pol3-t* mutation into strains YR50-1, RSY12, YR50-12, and YMG1, respectively. This was done by transformation of the cells with plasmid p171 (a gift from Michael Resnick, National Institute of Environmental Health Sciences, Research Triangle Park, NC), which contains a 2.2-kb *EcoRV-HindIII* fragment containing the *pol3-t* allele [15]. The cells were transformed with *HpaI*-linearized p171. Temperature-sensitive Ura⁺ colonies that contained the full-length *pol3-t* allele and a truncated *POL3* allele flanking the *URA3* gene were isolated. Ura⁻ temperature-sensitive strains carrying the *pol3-t* allele were selected after selection on medium containing 5-FOA [33].

2.3. Methyl Methanesulfonate (MMS) Survival Assay. Single colonies of strain RSY6 and its derivatives *mag1* Δ , *rad50* Δ , *pol3t*, *mag1* Δ *pol3t* and *rad50* Δ *pol3t* were inoculated into YPAD at 25° for 24 hours. Thereafter, cells were washed, resuspended in 5 mL of fresh YAPD at the concentration of 3×10^6 cells/mL and exposed to MMS for 4 hours at 30°. Then cells were washed twice, counted, and plated in YPAD at the concentration of 200 cells per plate. Plates were incubated at 25°C for 5 days.

2.4. Intrachromosomal Recombination Assay. All strains derived from RSY6 carry the same intrachromosomal recombination substrate as strain RSY6 [13]. This substrate consists of two *his3* alleles, one with a deletion at the 3' end and the other with a deletion at the 5' end, which share 400 bp of homology. These two alleles are separated by the *LEU2* marker and by the plasmid DNA sequence. An intrachromosomal recombination event between the two *his3* alleles leads to *HIS3* reversion and loss of *LEU2* [13]. To determine the frequency of spontaneous intrachromosomal recombination, single colonies were inoculated into 5 ml of SC-LEU, so that recombinants cannot grow, and incubated at 25° or 30° for 24 hours. Thereafter, cultures were washed twice and counted and appropriate numbers were plated onto SC and SC-HIS plates to determine the surviving fraction and the frequency of intrachromosomal recombination events, respectively.

Intrachromosomal recombination was measured following UV, γ -ray, and MMS exposure. For UV exposure single colonies were inoculated into SC-LEU at 25°C for 24 hours. Thereafter, cells were washed, resuspended in fresh SC-LEU for 4 hours at 30°C. 10 mL aliquots containing 3×10^7 cells/ml were irradiated in distilled water using a UV source at the dose rate of 3.5 ergs/m²/sec. The same number of cells were exposed to γ -rays using a ⁶⁰Co γ -ray source at 9.1 cGy per second [30, 34]. Following irradiation, cells were plated as described above. For MMS exposure, single colonies were inoculated into SC-LEU at 25° for 24 hours. Thereafter, cells were washed, resuspended in 5 mL of fresh SC-LEU at the concentration of 3×10^6 cells/ml and exposed to MMS for 4 hours at 30°. Then cells were washed, counted and plated as described. At the highest MMS dose only the wild type strain grew for one generation; at low doses all strains grew an average of 2-3 generations.

2.5. Gene Replacement by Homologous Recombination (Gene Targeting). The gene targeting events were determined in the RSY12 strain and its derivatives Y50-12, AGY38, and AGY39 which carry the complete deletion of the *URA3* gene [17]. The *EcoRI-HindIII* fragment from plasmid pJZ102 carrying the *LYS2* gene disrupted by *URA3* insertion was transformed in all the RSY12 derivative strains as previously described [16]. Transformants were selected on SC-URA plates and, then, replicated in SC-LYS medium. The frequency of homologous gene replacement was calculated as number of total *URA3*^{+*lys2*⁻ colonies $\times 10^{-4}$ transformed cells per μ g DNA. The number of transformed cells per μ g DNA}

was determined using the episomal plasmid YEplac195 as previously described [16]

2.6. Data Comparison and Statistical Analysis. Results were statistically analysed using the Student's *t*-test. Probabilities are shown as **P* < .05, ***P* < .01, ****P* < .001. The rate of DNA damage-induced recombination was extrapolated as follows: for each strain, we measured the number of recombination events induced for a range of increasing dosages in single experiment. From one experiment, we fitted the best-fit line to the data and took the slope of this line as the rate of induction. We used a student's *t* test to compare between individually extrapolated rate of induction values between strains (for the same DNA damaging agent).

3. Results

3.1. Epistatic Interaction between *mag1*, *rad50*, and *pol3* for MMS Sensitivity. The main lesion MMS produced in DNA is methylation, primarily producing 3-methyladenine (3MeA). 3MeA is mainly repaired by base excision repair (BER), but some lesions can be converted to DSBs which are repaired by nonhomologous end joining or homologous recombination [35–38]. It has previously been shown that *pol3* mutant cells are sensitive to MMS which is taken as evidence for involvement of DNA polymerase δ in the base excision repair pathway [6].

The 3MeA DNA glycosylase, encoded by the *MAG1* gene, has been shown to be very important for 3MeA removal from DNA [39, 40]. The *pol3-t* mutant is sensitive to the alkylating agent MMS as reported for other *pol3* mutants [6, 14]. The deletion of the *RAD50* gene also confers high sensitivity to MMS [41]. In the present study, we determined the epistasis of the MMS sensitivity of the *pol3-t* mutant with the base excision repair mutation *mag1* and the double strand break repair gene *rad50*. Previously, it has been shown that the *mag1* Δ and the *rad50* Δ mutations show a synergistic interaction with respect to MMS sensitivity implying that *MAG1* and *RAD50* act in distinct repair pathways [41]. Here the *pol3-t* mutant was more sensitive to MMS than wild type (Figure 1), and the double mutant *mag1* Δ *pol3-t* was more sensitive to MMS than each single mutant indicating that *MAG1* and *POL3* belong to different repair pathways (Figure 1(a)).

The *rad50* Δ mutant is very sensitive to MMS (Figure 1(b)). Moreover, the double mutant *rad50* Δ *pol3-t* showed the same sensitivity to MMS as the *rad50* Δ single mutant. This suggests that *RAD50* and *POL3* may belong to the same pathway for repairing MMS-induced lesions.

3.2. The Hyperrecombination Phenotype of *pol3-t* Is Abolished by Mutation of *RAD50*. We previously have shown that the *pol3-t* mutation causes a hyperrecombination phenotype in yeast that is partially dependent on *RAD52* and *RAD1* [14]. This suggests that replication slippage or a single-strand annealing pathway that is *RAD52* and *RAD1* independent could be responsible for the hyperrecombination phenotype

TABLE 2: Effect of RAD50 on the hyperrecombination phenotype of *pol3-t*.

Strain	Genotype	Intrachromosomal recombination ($\times 10^{-4}$)	
		25°C	30°C
RSY6	RAD, POL3	2.49 \pm 1.55	2.70 \pm 0.89
AGY30	RAD, <i>pol3-t</i>	36.8 \pm 12.66***	86.52 \pm 10.57***
YR50-1	<i>rad50</i> Δ , POL3	1.92 \pm 0.67	1.53 \pm 0.96
AGY34	<i>rad50</i> Δ , <i>pol3-t</i>	2.72 \pm 1.02	1.86 \pm 1.07

Single colonies of each strain were inoculated in SC-LEU and incubated for 24 hours at 25° or 30°C. Then, cells were washed and plated to determine the frequency of intrachromosomal recombination as described in the Materials and Methods. Results are the mean of 6 independent experiments \pm standard deviation. *** $P < .001$.

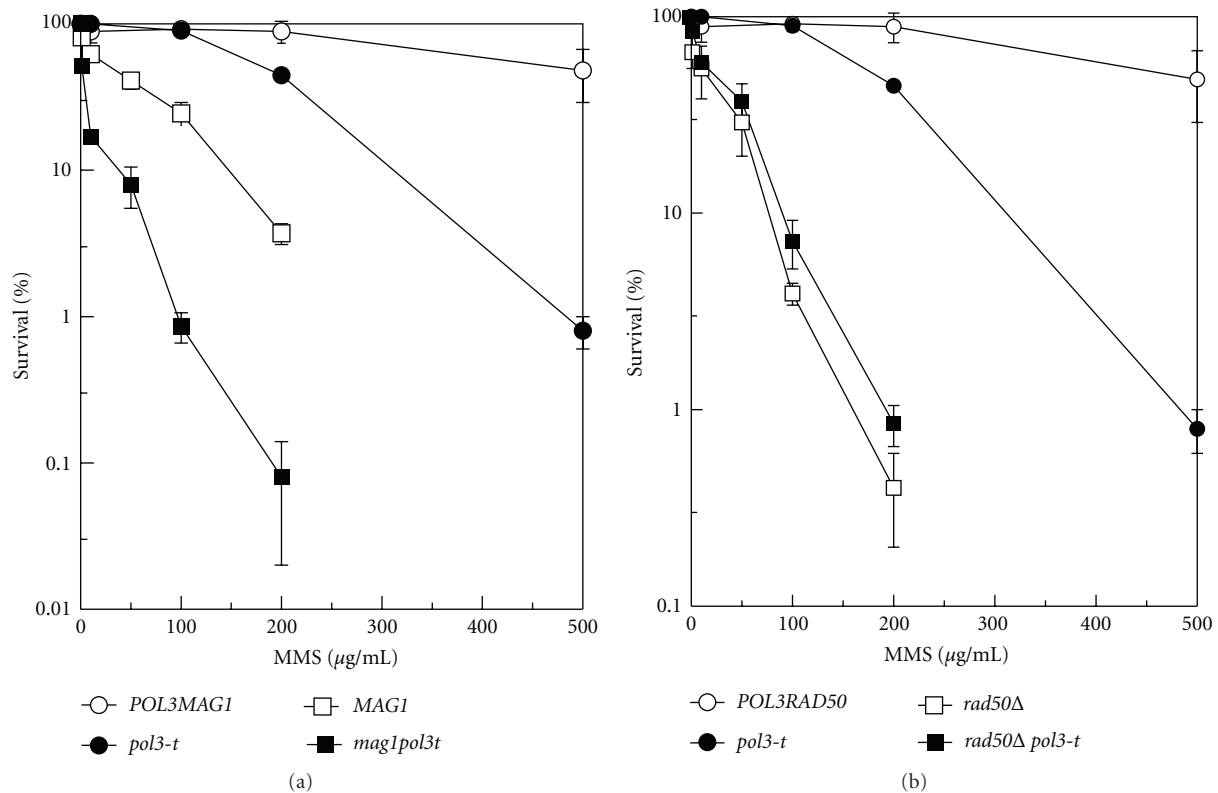


FIGURE 1: Epistasis interaction between *mag1* Δ and *pol3-t* (a); and *rad50* Δ and *pol3-t* (b). All strains were isogenic to RSY6. Single colonies of each strain were pregrown at 25°C for 24 hours and exposed to MMS for 4 hours at 30°C as reported in the materials and methods. Data are reported as the mean of six or more independent experiments \pm standard deviation.

[14]. The *RAD50* gene product is involved in DNA replication slippage between distant repeats [11, 42]. Moreover, the deletion of the *RAD50* gene in the *pol3-t* background decreases the frequency of excision of Tn5 [10]. To investigate the effect of *rad50* on *pol3-t*-mediated recombination, we constructed the haploid strain AGY34 which contains an intrachromosomal recombination substrate (see materials and methods). The *pol3-t* mutation confers a temperature-sensitive phenotype and growth arrest at 37°C; therefore we measured the effect of the *rad50* deletion mutation after growth at 25°C and 30°C [10]. Single colonies of RSY6, AGY30, YR50-1, and AGY34 were inoculated into SC-LEU

medium for 24 hours at 25°C and 30°C. During this period RSY6 (wild type) and YR50-1 (*rad50* Δ) underwent 4 to 5 cell divisions at both temperatures. AGY30 (*pol3-t*) and AGY34 (*rad50* Δ *pol3-t*) underwent 3 cell divisions at 25°C and 2 cell divisions at 30°C. In the *pol3-t* strain, intrachromosomal recombination increased 14-fold at 25°C and 32-fold at 30°C confirming that *pol3-t* confers a hyperrecombination phenotype (Table 2, [14]). In the *rad50* Δ background strain, the *pol3-t* mutation did not increase intrachromosomal recombination at either 25°C or 30°C (Table 2) demonstrating that the *rad50* deletion completely abolished the *pol3-t*-mediated hyperrecombination phenotype.

TABLE 3: Effect of *pol3-t* on homologous integration in *Saccharomyces cerevisiae*. Homologous gene targeting was measured by transformation of the plasmid pJZ102 digested with *EcoRI-HindIII* to release a *URA3* fragment that was flanked by the 5' and 3' ends of *LYS2* gene. Only *URA3lys2* colonies were counted as homologous integrants. In parentheses, the total *URA3lys2* colonies counted are reported. Data are reported as mean of at least 6 independent experiments \pm standard deviation.

Strain	Integration events/ μ g DNA per 10^4 transformed cells
RSY12 (<i>POL3RAD50</i>)	263.9 \pm 79.3 (4310)
AGY38 (<i>pol3-t</i>)	2936.8 \pm 769.8 (22247)*** ^(a)
YR50-12 (<i>rad50</i> Δ)	434.6 \pm 104.1 (7196)
AGY3 (<i>rad50</i> Δ <i>pol3-t</i>)	2780.4 \pm 445.5 (17416)*** ^{(a)(b)}

*** $p < .005$

^(a)Data were statistically compared to RSY12

^(b)Data were statistically compared to *rad50* Δ .

3.3. *The Elevated Frequency of Gene Replacement by Homologous Recombination of pol3-t Mutant Is Not RAD50 Dependent.* Integration of linear DNA fragment by homologous recombination into a chromosomal gene is thought to occur by two independent strand invasion events leading to the replacement of the chromosomal target with the DNA fragment [43, 44]. The homologous integration is reduced in the *rad1*, *rad51*, *rad52*, and *rad57* deletion mutant while it is unaffected in the *rad50* [16]. We, therefore, measured the homologous integration in the *pol3-t* and in the *rad50* Δ *pol3-t* mutant. The RSY12, AGY38, AGY39, and Y50-12 strains were transformed with the *URA3* fragment flanked by *lys2* sequence. As an homologous integration event leads to the replacement of chromosomal *LYS2* gene with the *URA3* fragment, the frequency of homologous integration was determined as number of *URA3*⁺*lys2*⁻ colonies $\times 10^{-4}$ transformed cells per μ g DNA.

In the *pol3-t* mutant, the frequency of homologous integration increased 11-fold as compared to the wild type (Table 3). In the double mutant *rad50* Δ *pol3-t*, the frequency has increased 10.5-fold as compared to the wild type indicating that the elevated level of integration is not *RAD50* dependent (Table 3). As previously reported, the *rad50* Δ mutation did not affect the frequency of homologous integration as compared to the wild type (Table 3).

3.4. *Effect of rad50 Deletion on MMS, UV, and γ -Ray Induced Intrachromosomal Recombination in the pol3-t Strain.* MMS, UV, and γ -rays induced intrachromosomal recombination in the *pol3-t* mutant [14] (Tables 4, 5, and 6). To further characterize the *pol3-t* phenotype, we looked at whether the *RAD50* deletion could also suppress mutagen-induced intrachromosomal recombination events in the *pol3-t* mutant. Single colonies of both YR50-1 (*rad50* Δ) and AGY34 (*rad50* Δ *pol3-t*) strains were grown at 25°C for 17 hours and then incubated at 30°C for 4 hours before MMS, UV, and γ -ray exposure after which survival and intrachromosomal events were scored for a range of doses of each mutagen. The rate of intrachromosomal recombination induction with

TABLE 4: Effect of *pol3-t* on MMS-induced intrachromosomal recombination in *RAD*⁺ and *rad50* Δ strains. Data corresponding to RSY6 and AGY30 were previously published [14]. Results are reported as the mean of five or more independent experiments \pm standard deviation. All strains are isogenic. The probabilities refer to the comparison between the exposure and the untreated control.

Strain	MMS (μ g/ml)	% Survival	Intrachromosomal recombination ($\times 10^{-4}$)
RSY6 (<i>POL3 RAD50</i>)	0	100	4.3 \pm 1.0
	10	89 \pm 15	9.3 \pm 1.5**
	100	92 \pm 8	39 \pm 6.6***
	200	89 \pm 15	51 \pm 14**
	500	48 \pm 19	102 \pm 25***
AGY30 (<i>pol3-t</i>)	0	100	17 \pm 4.6
	10	100 \pm 0.5	14 \pm 8.4
	100	90 \pm 0.5	30 \pm 4.9*
	200	45 \pm 1.5	60 \pm 28**
	500	0.8 \pm 0.6	117 \pm 2.8***
YR50-1 (<i>rad50</i> Δ)	0	100	1.8 \pm 0.6
	1	66 \pm 11	1.6 \pm 0.5
	10	54 \pm 16	2.4 \pm 0.8
	50	19 \pm 2.8	2.3 \pm 0.2
	100	3.9 \pm 0.5	3.6 \pm 0.6**
AGY34 (<i>rad50</i> Δ <i>pol3-t</i>)	0	100	1.8 \pm 0.2
	1	84 \pm 22	1.7 \pm 0.1
	10	58 \pm 14	1.7 \pm 0.6
	50	37 \pm 8.5	2.2 \pm 0.4
	100	7.2 \pm 2	3.5 \pm 0.1***
200	0.8 \pm 0.7	12.8 \pm 1.9***	

* $P < .05$, ** $P < .01$, *** $P < .001$.

dose for each experiment was found by linear regression; comparison of the induction rate between strains was made using student's *t*-test. To see if *pol3-t* strains are defective in DNA damage-induced intrachromosomal recombination, we compared the rate of recombination induction among strains. Then, we measured the number of recombination events induced for a range of increasing dosages in single experiment. From one experiment, we fitted the best-fit line to the data and took the slope of this line as the rate of induction. Each experiment was done in at least triplicate, and thus, for each mutagen and each strain there are at least three separate measures of the rate of induction with dose.

Survival and intrachromosomal recombination events were measured in wild type and *pol3-t* strains to 0, 10, 100, 200, and 500 μ g/mL MMS and in *rad50* Δ and *rad50* Δ *pol3-t* strains to 0, 1, 10, 50, 100, and 200 μ g/mL. 10 μ g/mL MMS induced a significant increase of intrachromosomal recombination in the wild type but none in each of the mutants (Table 4). Yet 100 μ g/ml MMS was the lowest dose used that significantly increased intrachromosomal recombination in each of the strains. The rate of intrachromosomal

TABLE 5: Effect of *pol3-t* on UV-induced intrachromosomal recombination in *RAD*⁺ and *rad50Δ* strains.

Strain	UV (J/m ²)	% survival	Intrachromosomal Recombination (×10 ⁻⁴)
RSY6 (<i>POL3 RAD50</i>)	0	100	6.2 ± 1.4
	5	77 ± 15	8.4 ± 1.9
	10	62 ± 23	10 ± 2.9
	100	41 ± 8.7	20 ± 6.2*
	500	27 ± 8.3	28 ± 5.6**
AGY30 (<i>pol3-t</i>)	0	100	14 ± 1.7
	5	69 ± 14	17 ± 3.4
	10	69 ± 11	13 ± 2.0
	100	57 ± 13	25 ± 6*
	500	39 ± 3.5	22 ± 3*
YR50-1 (<i>rad50Δ</i>)	0	100	1.2 ± 0.6
	5	68 ± 11	1.7 ± 0.6
	10	48 ± 28	1.5 ± 0.8
	100	39 ± 19	2.5 ± 1.5
	200	35 ± 17	1.3 ± 0.7
AGY34 (<i>rad50Δpol3-t</i>)	0	100	1.9 ± 0.8
	5	70 ± 10	2.0 ± 0.7
	10	71 ± 22	2.3 ± 0.9
	100	62 ± 17	4.3 ± 1.5*
	200	34 ± 9	4.5 ± 0.7**
	500	26 ± 12	4.8 ± 1.1**

See legend to Table 4.

recombination induction was 19.3 ± 1.9 ($\times 10^{-2}$ per $\mu\text{g/mL}$ MMS) in the wild type and 20.1 ± 1.3 ($\times 10^{-2}$ per $\mu\text{g/mL}$ MMS) in the *pol3-t*. The *rad50Δ* mutation resulted in a significantly lower induction rates of 1.3 ± 0.3 ($\times 10^{-2}$ per $\mu\text{g/mL}$ MMS) ($P < .005$) and was partially restored to the wild type level in the double mutant *rad50Δpol3-t* with 5.0 ± 1.1 ($\times 10^{-2}$ per $\mu\text{g/mL}$ MMS) ($P < .005$ when compared to *rad50Δ*). This suggests that RAD50 is required for MMS-induced intrachromosomal recombination more so in the wild type than in the *pol3-t* mutant background.

UV exposure also induced an increase in intrachromosomal recombination in each of the strains. A fluence of 100 J/m² induced a significant increase in each of the strains except the *rad50Δ* mutant for which a significant increase was not observed below 500 J/m² (Table 5). The intrachromosomal recombination induction rate was 42.1 ± 5.5 ($\times 10^{-3}$ per J/m² UV) in the wild type and 17.3 ± 3.9 ($\times 10^{-3}$ per J/m² UV) in the *pol3-t* strain. This rate was significantly lower in both the *rad50Δ* and *rad50Δpol3-t* strains which exhibited induction rates of 2.1 ± 0.4 and 3.8 ± 1.0 ($\times 10^{-3}$ per J/m² UV), respectively ($P < .005$ for both compared to wild type and *pol3-t*). This suggests that *rad50* had a reducing effect in the *POL3* as well as in the *pol3-t* mutant to UV-induced intrachromosomal recombination events (Table 5).

TABLE 6: Effect of *pol3-t* on γ -ray-induced intrachromosomal recombination in *RAD*⁺ and *rad50Δ* strains.

Strain	γ -Rays (Gy)	% survival	Intrachromosomal Recombination (×10 ⁻⁴)
RSY6 (<i>POL3 RAD50</i>)	0	100	2.4 ± 0.1
	50	65 ± 2.5	6.2 ± 0.9**
	500	29 ± 4.6	18 ± 2.3***
	1000	5 ± 2.4	39 ± 7.1***
AGY30 (<i>pol3-t</i>)	0	100	8.3 ± 0.5
	50	60 ± 11	12 ± 1.5*
	500	14 ± 2.5	23 ± 6.5*
YR50-1 (<i>rad50Δ</i>)	0	100	1.8 ± 0.24
	1	71 ± 13	2.9 ± 0.37**
	10	46 ± 0.8	3.9 ± 0.38**
AGY34 (<i>rad50Δpol3-t</i>)	0	100	1.1 ± 0.16
	1	69 ± 16	3.1 ± 0.97**
	10	35 ± 12	5.1 ± 2.51**
	50	5 ± 0.7	6.1 ± 0.8***

See legend to Table 4.

The *rad50Δ* and the *rad50Δpol3-t* strains are much more sensitive to γ -rays than the wild type and the *pol3-t* single mutant strains, respectively, yet little difference in γ -ray sensitivity was found among the *rad50Δ* and *rad50Δpol3-t* strains (Table 6). The *rad50Δ* phenotypic sensitivity to ionizing radiation is severe; the dose corresponding to 5%–10% survival, 50 Gy, is approximately 20x less than an equitoxic dose in the wild type and *pol3-t* strains. Because *rad50Δ* and *rad50Δpol3-t* strains exhibit extremely low survival to γ -rays at doses >50 Gy, the rate of intrachromosomal recombination from 0 and 50 Gy was compared between each of the four strains, a dose range at which recombination was found to sharply increase with dose. Within this dose range, the rate of intrachromosomal recombination in each of the strains was as follows: 79.3 ± 35.5 ($\times 10^{-3}$ per Gy) in wild type, 101.6 ± 46.9 ($\times 10^{-3}$ per Gy) in *pol3-t*, 69.5 ± 20.1 ($\times 10^{-3}$ per Gy) in *rad50Δ*, and 77.8 ± 22.6 ($\times 10^{-3}$ per Gy) in the *rad50Δpol3-t* double mutant. Thus between the range 0–50 Gy, all strains exhibited a similar rate of intrachromosomal recombination. The *pol3-t* toxicity to ionizing radiation is similar to wild-type, and thus the rate of γ -ray-induced recombination was compared between these strains between 50 and 1000 Gy, a range at which recombination is induced at a lower rate (Table 6). Here, the *pol3-t* strain showed a trend of a lower induction rate than that of wild type: 19.8 ± 4.3 versus 35.6 ± 13.2 ($\times 10^{-3}$ per Gy) ($P = .06$).

4. Discussion

We found that *pol3-t* was synergistic with *mag1* for MMS toxicity but epistatic with *rad50*. This suggests that *POL3*

may participate in the *RAD50* pathway for repair of MMS damage. *RAD50* is involved in processing the ends of a DSB [18]. The mechanism by which the MMS-induced lesions are converted to DSBs is not completely understood except that DSB repair-deficient mutants are also sensitive to MMS. In theory, it is possible that MMS damaged sites are converted into DSBs, and *POL3* is involved in their repair even though there is no published evidence for that. However, in our experiments the *pol3-t* mutant was not more sensitive to ionizing radiation that causes DSBs arguing against *POL3* involvement in DSB repair.

Recently, it has been shown that MMS does not induce DSBs in both yeast and mammalian cells [45]. The number of alkylated sites converted to single-strand breaks and DSBs, however, could be too few to be detected by their assay, but enough to require the involvement of the DSB repair pathway. The authors also suggested that the alkylation damage may stall the replication fork leading to the formation of a chicken foot structure which resembles a Holliday junction [45]. This may explain the reason why the DSB repair mutants that are also deficient in recombination are sensitive to MMS [45]. This would imply some involvement of *RAD50* in resolution of stalled replication forks, which may involve recombination [21, 22]. In fact, the gene products 46/47, the bacteriophage T4 homologs of Mre11/Rad50, are required for recombination-induced replication [23–25]. Furthermore, the Mre11 complex colocalizes with replication forks [46]. In this scenario, both *POL3* as well as *RAD50* may be involved in replication on the MMS-damaged template explaining their epistasis for MMS sensitivity.

We also found that the elevated level of intrachromosomal deletion recombination events in the *pol3-t* mutant is dependent on the *RAD50* gene. The *RAD50* protein is part of a complex that plays a major role in processing of DNA DSB ends [47]; therefore DNA DSB processing may be necessary for conferring the hyperrecombination phenotype of *pol3-t*. On the other hand, as discussed above for MMS toxicity, *RAD50* may be involved in recombinational resolution of replication forks stalled by DNA damage. In the presence of the *pol3-t* mutation, when replication is stalled at the restrictive temperature, such recombinational resolution may become important. We have previously shown that *pol3-t* causes a hyperrecombination phenotype dependent upon DNA replication [14]. If the second copy of the *HIS3* repeat is accidentally used as template for such resolution rather than the copy at which replication has stalled, the *pol3-t*-caused hyperrecombination phenotype may be mediated by *RAD50*. In a similar way for the involvement of *RAD50* in the *pol3-t*-mediated replication slippage at direct repeats within *LYS2* a possible replication function of *RAD50* has been proposed, rather than DSBs being involved in the slippage events [11]. We, indeed, found that the elevated frequency of homologous integration conferred by the *pol3-t* mutation was not affected by *RAD50*. This may indicate that the slow replication rate of the *pol3-t* mutant may favor homologous recombination events between the chromosomal DNA and an exogenous DNA fragment. Moreover, *RAD50* that is primarily involved in DSB processing did not affect this phenotype.

We found that the *rad50* mutant is almost completely deficient in MMS, and UV-induced recombination but no difference was observed in γ -rays-induced recombination. It has also previously been found that *RAD50* is involved in MMS but not UV-induced recombination between homologs in a diploid [48]. We have previously shown that intrachromosomal recombination between repeats is induced by site specific DSBs in G1, G2, and dividing cells [34]. A site-specific single strand break, however, induced recombination only in dividing but not in G1 or G2 arrested cells [34]. In a similar fashion, MMS, EMS, 4-NQO, and most but not all ionizing radiation-induced DNA damage were dependent on DNA replication for induction of intrachromosomal recombination [49]. This indicates that either replication turns the various DNA damages into DSBs, which then induces intrachromosomal recombination by single-strand annealing, or that recombination is induced by resolution of DNA-damaged replication forks as mentioned above. The latter explanation would be in agreement with *RAD50* being involved in DNA damage-induced resolution of stalled replication forks since most of the damage-induced recombination was *RAD50*-dependent similar to *pol3-t* induced recombination. At greater levels of DNA damage some DSBs may be formed accounting for the low level of recombination induction in the *rad50* mutant.

Interestingly, no difference was observed in γ -ray-induced recombination events. In the wild type the rate of γ -ray-induced recombination was observed to be biphasic with the number of events increasing sharply between 0 and 50 Gy, and at doses beyond 50 Gy the rate of recombination was substantially lower. Because the *rad50* Δ mutation is extra sensitive to ionizing radiation, the rate of recombination events was not scored at doses above 50 Gy. Between 0 and 50 Gy neither *rad50* Δ nor *pol3-t* mutations had an effect on the rate of γ -ray-induced recombination. It is possible that *rad50* Δ is involved in a second phase of γ -ray induced recombination at doses above 50 Gy or that the types of damage primarily produced by ionizing radiation are not repaired though the *RAD50* pathway. Either of such would explain why no difference in rate of intrachromosomal recombination was observed between 0 and 50 Gy.

In summary, it is unlikely that *POL3* is only involved in base excision repair. Furthermore, the epistasis for MMS sensitivity and the deficiency in DNA damage induced as well as complete block in *pol3-t* induced intrachromosomal recombination by *rad50* is in agreement with involvement of *RAD50* in repair by recombination resolution of stalled replication forks.

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