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 rad50Δ, 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 rad50Δ by examining their effect on spontaneous, MMS-, UV-, and ionizing radiation-induced intrachromosomal recombination. We found that rad50Δ 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, rad50Δ 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]. Polδ together with Polα and Polε performs essential functions required for DNA replication. Polα has a primase activity and is involved in initiation of both the leading and lagging strands [2]. Both Polδ and Polε can extend the primers formed by Polα [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].
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 methanesulfonate (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, YR50-12, YR50-15, 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 *ura3-52* 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 *mag1Δ, rad50Δ, pol3t, mag1Δpol3t* and *rad50Δ pol3t* were inoculated into

| Name      | Parent strain | Genotype                                                                 | Source          |
|-----------|---------------|---------------------------------------------------------------------------|-----------------|
| RSY6      |               | MATαura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3 ΔS′-pRS6-his3 ΔS′ | [13]            |
| YR50-1    | RSY6          | MATαura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3ΔS′-pRS6-his3ΔS′, rad50::hisG | Schiestl collection |
| AGY30     | RSY6          | MATαura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3ΔS′-pRS6-his3ΔS′, POL3-t | [14]            |
| YMG1      | RSY6          | MATαura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3ΔS′-pRS6-his3ΔS′, MAG1::hisG, pol3-t | Schiestl collection |
| AGY40     | YMG1          | MATαura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3ΔS′-pRS6-his3ΔS′, MAG1::hisG, pol3-t, rad50::hisG | This study      |
| AGY34     | YR50-1        | MATαura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3ΔS′-pRS6-his3ΔS′, POL3-t, rad50::hisG | This study      |
| RSY12     |               | MATαleu2-3,112 his3-11,15 URA3::HIS3                                     | [15]            |
| YR50-12   | RSY12         | MATαleu2-3,112 his3-11,15 URA3::HIS3, rad50::LEU2 | [16]            |
| AGY38     | RSY12         | MATαleu2-3,112 his3-11,15 URA3::HIS3, pol3-t | [17]            |
| AGY39     | YR50-12       | MATαleu2-3,112 his3-11,15 URA3::HIS3-pol3-t, rad50::LEU2 | This study      |
YPAD at 25°C for 24 hours. Thereafter, cells were washed, resuspended in 5 mL of fresh YPAD at the concentration of $3 \times 10^7$ cells/mL and exposed to MMS for 4 hours at 30°C. 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 400bp 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 $3 \times 10^7$ 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 $3 \times 10^7$ cells/ml and exposed to MMS for 4 hours at 30°C. 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 URA3LYS2− colonies $\times 10^{-4}$ transformed cells per μg DNA. The number of transformed cells per μg 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 mag1Δ and the rad50Δ 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 mag1Δpol3-t was more sensitive to MMS than each single mutant indicating that MAG1 and POL3 belong to different repair pathways (Figure 1(a)).

The rad50Δ mutant is very sensitive to MMS (Figure 1(a)). Moreover, the double mutant rad50Δpol3-t showed the same sensitivity to MMS as the rad50Δ single mutant. This suggests that RAD50 and POL3 may belong to the same pathway for repairing MMS-induced lesions.

3.2. The Hyperecombination Phenotype of pol3-t Is Abolished by Mutation of RAD50. We previously have shown that the pol3-t mutation causes a hyperecombination 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 hyperecombination phenotype [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 (rad50Δ) underwent 4 to 5 cell divisions at both temperatures. AGY30 (pol3-t) and AGY34 (rad50Δpol3-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 rad50Δ 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.

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

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

| Strain     | Genotype           | Intrachromosomal recombination (×10⁻⁴) |
|------------|--------------------|---------------------------------------|
|            |                    | 25°C       | 30°C       |
| RSY6       | RAD, POL3          | 2.49 ± 1.55 | 2.70 ± 0.89 |
| AGY30      | RAD, pol3-t        | 36.8 ± 12.66 *** | 86.52 ± 10.57 *** |
| YR50-1     | rad50Δ, POL3       | 1.92 ± 0.67 | 1.53 ± 0.96 |
| AGY34      | rad50Δ, pol3-t     | 2.72 ± 1.02 | 1.86 ± 1.07 |

Single colonies of each strain were inoculated in SC-LEU and incubated for 24 hours at 25°C 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 ± standard deviation. ***P < .001.
Table 3: Effect of pol3-t on homologous integration in Saccharomyces cerevisiae. Homologous gene targeting was measured by transformation of the plasmid pTZ102 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 ± standard deviation.

| Strain          | Integration events/µg DNA per 10^6 transformed cells |
|-----------------|-------------------------------------------------------|
| RSY12 (POL3RAD50)| 263.9 ± 79.3 (4310)                                   |
| AGY38 (pol3-t)  | 2936.8 ± 769.8 (22247)**/a)                         |
| YR50-12 (rad50Δ)| 434.6 ± 104.1 (7196)                                  |
| AGY3 (rad50Δpol3-t) | 2780.4 ± 445.5 (17416)***/(a)/(b)               |

**/P < .005
(a) Data were statistically compared to RSY12
(b) Data were statistically compared to rad50Δ.

homologous integration in the pol3-t and in the rad50Δpol3-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 URA3lys2 2− colonies ×10−4 transformed cells per µg 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 rad50Δpol3-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 rad50Δ 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 whether the RAD50 deletion could also suppress mutagen-induced intrachromosomal recombination events in the pol3-t mutant. Single colonies of both YR50-1 (rad50Δ) and AGY34 (rad50Δpol3-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 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 µg/mL MMS and in rad50Δ and rad50Δpol3-t strains to 0, 1, 10, 50, 100, and 200 µg/mL. 10 µg/mL MMS induced a significant increase of intrachromosomal recombination in the wild type but none in each of the mutants (Table 4). Yet 100 µg/mL MMS was the lowest dose used that significantly increased intrachromosomal recombination in each of the strains. The rate of intrachromosomal recombination induction was 19.3 ± 1.9 (×10−2 per µg/mL MMS) in the wild type and 20.1 ± 1.3 (×10−2 per µg/mL MMS) in the pol3-t. The rad50Δ mutation resulted in a significantly lower induction rates of 1.3 ± 0.3 (×10−2 per µg/mL MMS) (P < .005) and was partially restored to the wild type level in the double mutant rad50Δpol3-t with 5.0 ± 1.1 (×10−2 per µg/mL MMS) (P < .005 when compared

| Strain          | MMS (µg/ml) | % Survival | Intrachromosomal recombination (×10^−4) |
|-----------------|-------------|------------|----------------------------------------|
| RSY6 (POL3 RAD50)| 0           | 100        | 4.3 ± 1.0                              |
|                 | 10          | 89 ± 15    | 9.3 ± 1.5**                            |
|                 | 200         | 89 ± 15    | 51 ± 14**                              |
|                 | 500         | 48 ± 19    | 102 ± 25**                             |
| AGY30 (pol3-t)  | 0           | 100        | 17 ± 4.6                               |
|                 | 10          | 100 ± 0.5  | 14 ± 8.4                               |
|                 | 200         | 45 ± 1.5   | 60 ± 28**                              |
|                 | 500         | 0.8 ± 0.6  | 117 ± 2.8***                           |
| YR50-1 (rad50Δ) | 0           | 100        | 1.8 ± 0.6                              |
|                 | 10          | 66 ± 11    | 1.6 ± 0.5                              |
|                 | 50          | 19 ± 2.8   | 2.3 ± 0.2                              |
|                 | 100         | 3.9 ± 0.5  | 3.6 ± 0.6**                            |
|                 | 200         | 0.4 ± 0.2  | 4.2 ± 0.8***                           |
| AGY34 (rad50Δpol3-t) | 0     | 100        | 1.8 ± 0.2                              |
|                 | 10          | 84 ± 22    | 1.7 ± 0.1                              |
|                 | 50          | 37 ± 8.5   | 2.2 ± 0.4                              |
|                 | 100         | 7.2 ± 2    | 3.5 ± 0.1***                           |
|                 | 200         | 0.8 ± 0.7  | 12.8 ± 1.9***                          

*P < .05, **P < .01, ***P < .001.
Table 5: Effect of pol3-t on UV-induced intrachromosomal recombination in RAD+ and rad50Δ strains.

| Strain          | UV (J/m²) | % survival | Intrachromosomal Recombination (x10^-4) |
|-----------------|-----------|------------|----------------------------------------|
| RSY6 (POL3 RAD50) | 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 (pol3-t)  | 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*                                |
| YRS0-1 (rad50Δ) | 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                              |
|                 | 500       | 16 ± 9     | 2.6 ± 0.2**                            |
| AGY34 (rad50Δpol3-t) | 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.

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 (x10^-3 per J/m² UV) in the wild type and 17.3 ± 3.9 (x10^-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 (x10^-3 per J/m² UV), respectively (P < .005 for both compared to wild type and pol3-t). This suggests that rad50Δ had a reducing effect in the POL3 as well as in the pol3-t mutant to UV-induced intrachromosomal recombination events (Table 5).

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 (x10^-3 per Gy) in wild type, 101.6 ± 46.9 (x10^-3 per Gy) in pol3-t, 69.5 ± 20.1 (x10^-3 per Gy) in rad50Δ, and 77.8 ± 22.6 (x10^-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 (x10^-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 alklylation 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, involve recombination [21, 22]. In resolution of stalled replication forks, which may involve recombination [21, 22]. In resolution of stalled replication forks, which may involve recombination [21, 22].
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 rad50Δ 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 rad50Δ nor pol3-t mutations had an effect on the rate of γ-ray-induced recombination. It is possible that rad50Δ 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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