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Characterization of the Hyperrecombination Phenotype of the *pol3-t* Mutation of *Saccharomyces cerevisiae*

Alvaro Galli,* Tiziana Cervelli* and Robert H. Schiestl†,1

Laboratory of Gene and Molecular Therapy, Institute of Clinical Physiology, CNR, 56124 Pisa, Italy and †Department of Pathology and Environmental Health, UCLA School of Medicine, Los Angeles, California 90095

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ABSTRACT

The DNA polymerase δ (Pol3p/Cdc2p) allele *pol3-t* of *Saccharomyces cerevisiae* has previously been shown to increase the frequency of deletions between short repeats (several base pairs), between homeologous DNA sequences separated by long inverted repeats, and between distant short repeats, increasing the frequency of genomic deletions. We found that the *pol3-t* mutation increased intrachromosomal recombination events between direct DNA repeats up to 36-fold and interchromosomal recombination 14-fold. The hyperrecombination phenotype of *pol3-t* was partially dependent on the Rad52p function but much more so on Rad1p. However, in the double-mutant *rad1*Δ *rad52*Δ, the *pol3-t* mutation still increased spontaneous intrachromosomal recombination frequencies, suggesting that a Rad1p Rad52p-independent single-strand annealing pathway is involved. UV and γ-rays were less potent inducers of recombination in the *pol3-t* mutant, indicating that Pol3p is partly involved in DNA-damage-induced recombination. In contrast, while UV- and γ-ray-induced intrachromosomal recombination was almost completely abolished in the *rad52* or the *rad1 rad52* mutant, there was still good induction in those mutants in the *pol3-t* background, indicating channeling of lesions into the above-mentioned Rad1p Rad52p-independent pathway. Finally, a heterozygous *pol3-t/POL3* mutant also showed an increased frequency of deletions and MMS sensitivity at the restrictive temperature, indicating that even a heterozygous polymerase δ mutation might increase the frequency of genetic instability.

RECOMBINATION between repeated DNA sequences can occur in meiosis and in mitosis (Petes and Hill 1988; Klein 1995). Mitotic recombination between DNA repeats on the same chromosome, called intrachromosomal recombination, can lead to deletion of sequences located between the repeats, to gene conversion events that retain the duplication, or to triplications (Klein 1995). Genome rearrangements associated with recombination between homologous sequences can cause genetic disease and cancer and they increase in frequency by exposure to cancer-causing chemicals (Bishop and Schiestl 2000, 2001). It is thus important to identify the genetic and environmental factors leading to an increased frequency of such rearrangements, as well as to study the interaction between these factors.

Homologous intrachromosomal recombination events between duplicated sequences resulting in deletions may occur by several different mechanisms, such as intrachromatid exchange, single-strand annealing (SSA), one-sided invasion, unequal sister chromatid exchange, or sister chromatid conversion (Schiestl *et al.* 1988; Haber 1992; Belmaaza and Chartrand 1994; Galli and Schiestl 1995). Studies were previously carried out on the mechanism of reversion of a duplication of a 400-bp internal fragment of the *HIS3* gene separated by the *LEU2* gene (Schiestl *et al.* 1988). Intrachromatid exchange occurs as reciprocal crossing over between the direct repeats, which leaves a single copy of the gene on the chromosome and on the excised DNA fragment bearing the second copy of the gene. Schiestl *et al.* investigated the contribution of this mechanism to the frequency of such intrachromosomal recombination events by placing an origin of replication onto the integrated plasmid to recover both reciprocal products of an intrachromatid crossing-over event (Schiestl *et al.* 1988). They found that only a minority of events (∼1%) could be explained by this mechanism. With a different system that forced amplification of the excised circle, Santos-Rosa and Aguilera (1994) found that <10% of the deletion events produced circles. These results indicate that the majority of deletion events do not happen by intrachromatid crossing over, but rather by a nonconservative mechanism. SSA is initiated by a DNA double-strand break (DSB) in the nonhomologous region between the repeats. DNA degradation of single strands from the exposed 5′ ends of the DSB leads to single-strand regions that can anneal once the degradation has proceeded to the repeated sequences. The 3′ tails are processed and nicks are ligated, giving rise to a deletion. Another mechanism yielding deletion events is one-sided invasion, which is initiated by a DSB in one

[1]*Corresponding author:* UCLA Schools of Medicine and Public Health 71-295 CHS, 650 Charles E. Young Dr. S., Los Angeles, CA 90095. E-mail: botayde@mednet.ucla.edu

of the duplicated homologous sequences followed by 5′–3′ degradation (Belmaaza and Chartrand 1994). Invasion of the 3′ single strand occurs in the homologous region, leading to D-loop formation and to DNA synthesis. Resolution occurs by continuation of 5′ degradation, single-strand nick formation, and DNA repair synthesis.

Intrachromosomal recombination leading to deletions can also be explained by recombination between sister chromatids as unequal sister chromatid exchange (SCEs) or sister chromatid conversion. Unequal SCEs give rise to a duplication of the disrupting sequence (Schiestl *et al.* 1988; Galli and Schiestl 1995). The contribution of SCE events was determined by assaying for reciprocal products (Schiestl *et al.* 1988). Only ~4% of the recombination events gave such a triplication. This suggests that the majority of events are not due to unequal SCEs.

Intrachromatid exchange, SSA, and one-sided invasion can take place in any phase of the cell cycle, including G_1. SCE and sister chromatid conversion events, on the other hand, require the presence of the sister chromatid and thus they can occur in the S-phase or in G_2 but not in G_1. Intrachromosomal deletion recombination events are induced by a site-specific DSB in G_1 and G_2 to the same extent. Moreover, DNA single-strand breaks induce intrachromosomal deletion events in dividing but not in cell-cycle-arrested cells (Galli and Schiestl 1998b). This suggests that DNA DSBs are involved and that SSA is the main mechanism by which intrachromosomal deletion events occur (Galli and Schiestl 1998b). Mutations in *RAD1*, *RAD10*, and *RAD52* are involved in these intrachromosomal deletion events (Schiestl and Prakash 1988, 1990) and Rad1p has been shown on a molecular level to catalyze the excision of the nonhomologous DNA between the recombining duplicated alleles needed for the SSA pathway (Fishman-Lobell and Haber 1992; Ivanov and Haber 1995).

Several mutants with elevated spontaneous intrachromosomal recombination frequencies have been isolated in *Saccharomyces cerevisiae* (Aguilera and Klein 1988; Klein 1995). Among them, an allele of *CDC2/POL3*, which encodes the catalytic subunit of the DNA polymerase δ, increases deletion events but not gene conversions (Aguilera and Klein 1988). Polδp, together with Polαp and Polεp, is an essential function and required for DNA replication. Polαp has a primase activity and is involved in initiation of both the leading- and the lagging-strand syntheses (Brooks and Dumas 1989). Both Polδp and Polεp can extend the primers formed by Polαp (Burgers 1991; Podust and Hubscher 1993).

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 increases intrachromosomal deletion recombination between short repeats of several base pairs separated by long inverted repeats (Gordenin *et al.* 1992). The molecular analysis of the recombinants of the excision events of the transposon indicates that DNA replication slippage most likely is responsible for these deletion events (Tran *et al.* 1995; Gordenin and Resnick 1998). In agreement with this model, it has been shown that *pol3* mutations increase the frequency of additions and/or deletions of units of microsatellites (defined as repeat units from 1 to 13 bp) as well as minisatellites (>15 bp; Tran *et al.* 1995, 1996, 1999; Kokoska *et al.* 1998). Furthermore, the frequency of deletions between distant short repeats within the *LYS2* or the *CAN1* genes is also increased many fold (Tran *et al.* 1995; Kokoska *et al.* 2000). Finally, it has been shown that the same mutator phenotype as observed in the *pol3* mutations exists after repression of the *POL3* gene, indicating that the mutator phenotype may be due to low levels of Pol3p rather than to any other faulty effect of the Pol3p mutant proteins.

Here we report the effect of the temperature-sensitive allele *pol3-t* on intrachromosomal deletion and interchromosomal recombination, reverse and forward mutation. Moreover, we studied the influence of Rad1p and Rad52p in the *pol3-t* background to characterize the genetic control of intrachromosomal recombination. Finally, to better understand the role of DNA polymerase δ on DNA-damage-induced recombination, we also studied the effects of Rad1p and Rad52p on UV-, γ-ray-, and methyl methanesulfonate (MMS)-induced intrachromosomal deletion recombination in the *pol3-t* background.

MATERIALS AND METHODS

Media, genetic, and molecular techniques: Complete media (YPAD), synthetic complete (SC), and drop-out (SD) media were prepared according to standard procedures (Kaiser *et al.* 1994). Magic Column (Promega, Madison, WI) was used for preparation of small-scale DNA. Other general molecular techniques were carried out according to Maniatis *et al.* (1989). Yeast transformation was performed using the procedure described in Gietz *et al.* (1992, 1995).

Yeast strains: The names and genotypes of the strains of *S. cerevisiae* used are listed in Table 1. Because *pol3-t* confers a temperature-sensitive phenotype, all *pol3-t* strains were grown at 25° (Gordenin *et al.* 1992). Strains TCY1 and TCY2 were constructed by transformation of strains POL-DM and pol3-t-DM with plasmid pRS6, which contains an internal fragment of *his3* and a *LEU2* marker (Schiestl *et al.* 1988). This generates duplication within the *HIS3* gene, resulting in two incomplete *his3* alleles (see below).

Strains TCY3 and TCY4, carrying a deletion from position +40 to +3211 of *RAD1*, were constructed by two-step gene replacement using the *Eco*RI-*Sal*I fragment of plasmid pR1.6 (kindly provided by Louise Prakash; Saparbaev *et al.* 1996) and subsequent 5-fluoroorotic acid (5-FOA) selection (Boeke *et al.* 1984). Strains AGY30, AGY31, AGY34, and AGY35 were constructed by introducing the *pol3-t* mutation into strains RSY6, YR1-16, and Y433. This was done by transformation of the cells with plasmid p171 (a gift from Mike Resnick, National Institute of Environmental Health Sciences, Research Triangle

Park, NC), which contains a 2.2-kb *Eco*RV-*Hin*dIII fragment containing the *pol3-t* allele (Kokoska *et al.* 1998). The cells were transformed with *Hpa*I-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 just the *pol3-t* allele were selected after selection on medium containing 5-FOA (Kokoska *et al.* 1998). Strains AGY32 and AGY33 carrying the *rad52-9* deletion (henceforth called *rad52*Δ) were constructed by digestion of plasmid pSM22 (from David Schield and R. Mortimer via Louise Prakash) with *Bam*HI and transformation of yeast cells with the *Bam*HI fragment in which the *Bgl*II-*Cla*I fragment in the open reading frame of the *RAD52* gene had been replaced by a *Bam*HI-*Cla*I fragment containing the *URA3* gene (Schiestl and Prakash 1990).

Diploid strains AGY36 and AGY37, isogenic to RS112, were constructed by mating AGY30 with AGY35 and RSY6 with AGY35, respectively.

Recombination assays: All strains used carry the same intrachromosomal recombination substrate as strain RSY6 (Schiestl *et al.* 1988). 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 leads to *HIS3* reversion and loss of *LEU2* (Schiestl *et al.* 1988). These two copies readily undergo intrachromosomal recombination, resulting in wild-type *HIS3* at a frequency of $\sim 10^{-4}$ (Schiestl *et al.* 1988). Diploid strains RS112 and AGY36 are also heteroallelic for *ade2-40* and *ade2-101*. An interchromosomal gene-conversion event produces *ADE2* reversions.

To determine the frequency of spontaneous intrachromosomal recombination, single colonies were inoculated into 5 ml of SC-LEU and incubated at 25° or 30° for 17 hr. 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, respectively. Single colonies of the diploid strains RS112 and AGY36 were incubated as above and in addition plated onto SC-ADE plates to determine the frequency of interchromosomal gene conversion. Plates were incubated at 25° for 4 days and colonies were counted thereafter. All *HIS3* and *ADE2* recombinants were checked for the presence of the *pol3-t* allele by replica plating and incubation at 37°.

Intrachromosomal recombination was also measured following UV, γ-rays, and MMS exposure. For UV exposure, single colonies were inoculated into SC-LEU at 25° for 17 hr. Thereafter, cells were washed and resuspended in fresh SC-LEU for 4 hr at 30°. Aliquots of 10 ml containing 3×10^7 cells/ml were irradiated in distilled water using a UV source at the dose rate of 3.5 erg/m^2/sec. The same number of cells were exposed to γ-rays using a ^{60}Co γ-ray source at 9.1 cGy/sec (Galli and Schiestl 1995, 1998b). Following irradiation, cells were plated as described above. For MMS exposure, single colonies were inoculated into SC-LEU at 25° for 17 hr. 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 hr at 30°. Then cells were washed, counted, and plated as described.

Reverse and forward mutation assay: To measure the spontaneous frequency of reverse mutations at *ilv1-92* and *arg4-3*, single colonies of RSY6 and AGY30 were inoculated into 5 ml YPAD and incubated for 17 hr at 25° or 30°. Then cells were washed and counted and appropriate numbers were plated onto SC, SC-ILV, and SC-ARG to score for the surviving fraction and mutants. Plates were incubated at 25° until colonies were formed.

The spontaneous frequency of forward mutation was determined as follows: single colonies of RSY6 (ARG4) and AGY30 (ARG4) were inoculated in 5 ml YPAD and incubated for 17 hr at 25° or 30°. Then cells were washed and counted and appropriate numbers were plated onto SC and SC-ARG + CAN (60 mg/liter) to score for the surviving fraction and mutants. Plates were incubated at 25° until colonies formed.

Determination of the effect of cell division on the recombination phenotype in the *pol3-t* mutant: We tested the effect of cell division on the recombination phenotype of *pol3-t* after growth at 25° and 30°. Single colonies of AGY30 and RSY6 were grown in SC-LEU at 25° for 20 hr. Cells were washed and inoculated for 5 hr in SC-URA to achieve cell-cycle arrest at G_0/G_1 since they carry the *ura3-52* allele (Galli and Schiestl 1995). Cell-cycle arrest was checked by counting unbudded cells under the microscope as previously described (Galli and Schiestl 1995). A total of 250–300 cells were counted per tube and 96.1 ± 0.7% were unbudded. Thereafter, cell cultures were divided into two aliquots; one aliquot was kept in SC-URA medium at 25° and the other one was incubated at 30° for 24 hr. Intrachromosomal recombination was measured at the 0 time point and after 24 hr of incubation.

Data comparison and statistical evaluation: The data were compared either as fold induction compared to the control or as "change in average frequency," which indicates the number of recombination events after exposure to a certain dose of a genotoxin after subtraction of the spontaneous frequency (Kadyk and Hartwell 1992, 1993; Galli and Schiestl 1998a; Paulovich *et al.* 1998). Results were statistically analyzed using the Student's *t*-test.

RESULTS

Effect of *pol3-t* on spontaneous mitotic recombination: To investigate effects of *pol3-t* on mitotic recombination we constructed the haploid strains TCY1, TCY2, and AGY30 and the diploid strain AGY36 (Table 1). All these strains contain an intrachromosomal recombination substrate that resulted from integration of plasmid pRS6 at the *HIS3* locus (see materials and methods; Schiestl *et al.* 1988). Intrachromosomal recombination between the two *his3* alleles, which share 400 bp of homology, leads to *HIS3* reversion and loss of *LEU2* (Schiestl *et al.* 1988). The diploid strains RS112 and AGY36 are heteroallelic for *ade2* and can also be used to measure interchromosomal recombination events (Table 1). The *pol3-t* mutation confers a temperature-sensitive phenotype and growth arrest at 37°; thus, we studied effects of *pol3-t* mutation on mitotic recombination after growth at 25° and 30° (Gordenin *et al.* 1992). Single colonies of TCY1, TCY2, RSY6, AGY30, RS112, and AGY36 were incubated in SC-LEU for 17 hr at 25° and 30°. During this incubation period, TCY1, RSY6, and RS112 underwent four to five cell divisions at both temperatures. TCY2, AGY30, and AGY36 underwent three to four cell divisions at 25° and two to four cell divisions at 30°. Appropriate aliquots were plated and incubated. *HIS3 leu2* colonies revealed deletion recombination frequencies. At 25°, *pol3-t* increased intrachromosomal recombination 8-fold in the diploid strain AGY36, 15- and 4-fold, respectively, in the haploid strains AGY30 and TCY2, and, at 30°, 36-fold in AGY36,

TABLE 1

***S. cerevisiae* strains**

Name	Parent strain	Genotype	Source
RSY6		*MAT***a** *ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5′-pRS6-his3Δ3′*	Schiestl *et al.* (1988)
Y433		*MATα ura3-52 leu2-Δ98 ade 2-101 ilv1-92 his3-Δ200 lys2-801*	Michael Snyder
RS112		*MAT***a** *ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5′-pRS6-his3Δ3′ LYS2* *MATα ura3-52 leu2-Δ98 TRP5 ade 2-101 ilv1-92 ARG4 his3-Δ200 lys2-801*	Schiestl *et al.* (1988)
POL-DM		*MATα lys2-Tn5-13 ura3-Δ1 leu2-2 trp1-Δ1*	Gordenin *et al.* (1992)
pol3t-DM		*MATα lys2-Tn5-13 ura3-Δ1 leu2-2 trp1-Δ1 pol3-t*	Gordenin *et al.* (1992)
YR1-16	RSY6	*MAT***a** *rad1Δ::HISG ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5′-pRS6-his3Δ3′*	Saparbaev *et al.* (1996)
YR52-2	RSY6	*MAT***a** *rad52Δ::URA3 ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5′-pRS6-his3Δ3′*	Saparbaev *et al.* (1996)
YR1-18	RSY6	*MAT***a** *rad1Δ::HISG rad52Δ::URA3 ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5′-pRS6-his3Δ3′*	Saparbaev *et al.* (1996)
TCY1	POL-DM	*MATα lys2-Tn5-13 ura3-Δ1 leu2-2 trp1-Δ1 his3Δ5′-pRS6-his3Δ3′*	This study
TCY2	pol3t-DM	*MATα lys2-Tn5-13 ura3-Δ1 leu2-2 trp1-Δ1 pol3-t his3Δ5′-pRS6-his3Δ3′*	This study
TCY3	POL-DM	*MATα lys2-Tn5-13 rad1Δ::HISG ura3-Δ1 leu2-2 trp1-Δ1 his3Δ5′-pRS6-his3Δ3′*	This study
TCY4	pol3t-DM	*MATα lys2-Tn5-13 rad1Δ::HISG ura3-Δ1 leu2-2 trp1-Δ1 pol3-t his3Δ5′-pRS6-his3Δ3′*	This study
AGY30	RSY6	*MAT***a** *ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5′-pRS6-his3Δ3′ pol3-t*	This study
AGY31	RSY6	*MAT***a** *rad1Δ::HISG ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5′-pRS6-his3Δ3′ pol3-t*	This study
AGY32	RSY6	*MAT***a** *rad52Δ::URA3 ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5′-pRS6-his3Δ3′ pol3-t*	This study
AGY33	RSY6	*MAT***a** *rad1Δ::HISG rad52Δ::URA3 ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5′-pRS6-his3Δ3′ pol3-t*	This study
AGY35	Y433	*MATα ura3-52 leu2Δ98 ade 2-101 ilv1-92 his3Δ200 lys2-801 pol3-t*	This study
AGY36	RS112	*MAT***a** *ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5′-pRS6-his3Δ3′ LYS2 pol3-t* *MATα ura3-52 leu2Δ98 TRP5 ade 2-101 ilv1-92 ARG4 his3Δ200 lys2-801 pol3-t*	This study
AGY37	RS112	*MAT***a** *ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5′-pRS6-his3Δ3′ LYS2 POL3* *MATα ura3-52 leu2Δ98 TRP5 ade 2-101 ilv1-92 ARG4 his3Δ200 lys2-801 pol3-t*	This study

22-fold in AGY30, and 18-fold in TCY2 (Table 2). The *pol3-t* mutation did not significantly increase the frequency of interchromosomal recombination at 25° in the diploid strain AGY36, whereas it caused a significant 14-fold increase at 30°.

Effect of the *pol3-t* mutation on spontaneous mutation frequencies: Another temperature-sensitive mutant of DNA polymerase δ gene *CDC2*, named *hpr6*, has been shown to have both hyperrecombination and mutator phenotypes (Aguilera and Klein 1988). In addition, it has been shown that other *pol3* alleles increase the mutation frequency at different loci (Morrison and Sugino 1994; Giot *et al.* 1997; Kokoska *et al.* 2000). Therefore, we tested the effects of *pol3-t* on spontaneous mutation frequencies in our strain backgrounds. As shown in Table 3, the frequency of *ILV1* reverse mutation increased in AGY30 6-fold at 25° and 8-fold at 30°. At 25°, *pol3-t* did not affect the frequency of *ARG4* revertants, whereas it caused a small but significant 3-fold increase in reversion frequency at 30° (Table 3). The frequency of forward mutation, determined as frequency of canavanine-resistant ($can1^R$) mutants, increased 18-fold at 25° and 32-fold at 30° in the *pol3-t* strain (Table 3). Thus, *pol3-t*, like other *pol3* mutants, causes both hyperrecombination and mutator phenotypes.

Since *pol3-t* had a more pronounced effect on intrachromosomal recombination than on interchromosomal recombination, we decided to further focus our study on intrachromosomal recombination.

Dependence of the *pol3-t* hyperrecombination phenotype on DNA replication: In yeast, mRNA transcript levels of CDC2/POL3 increase at the boundary of the G_1/S phase of the cell cycle (Wang 1991) and return to a low level during or after the S-phase (Campbell and Newlon 1991).

TABLE 2

Effect of *pol3-t* on spontaneous intrachromosomal and interchromosomal recombination frequencies

		Intrachromosomal recombination ($\times 10^{-4}$)		Interchromosomal recombination ($\times 10^{-5}$)	
Strain	Genotype	25°	30°	25°	30°
RS112	*POL3*	0.74 ± 0.1	0.86 ± 0.09	0.78 ± 0.48	0.34 ± 0.24
AGY36	*pol3-t*	6.2 ± 1.5**	31 ± 0.41***	1.1 ± 0.21	4.7 ± 0.83***
RSY6	*POL3*	2.1 ± 1.4	3.5 ± 0.87		
AGY30	*pol3-t*	32 ± 16**	76 ± 14***		
TCY1	*POL3*	2.5 ± 0.5	2.4 ± 0.99		
TCY2	*pol3-t*	11 ± 6.0*	42 ± 15**		

Results are reported as the mean of five or more experiments ± standard deviation. Strain RS112 is isogenic with AGY36, RSY6 with AGY30, and TCY1 with TCY2. The probabilities refer to the comparison between the *POL3* strain and the *pol3-t* mutant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

pol3-t strains showed a pronounced hyperrecombination phenotype following growth at the semipermissive temperature of 30°. We tested the effect of cell division on the hyperrecombination phenotype of *pol3-t* after growth at 25° and 30° (see MATERIALS AND METHODS). Intrachromosomal recombination was measured at the 0 time point and after 24 hr of incubation. At time 0, the frequency of intrachromosomal recombination was $2.34 \pm 0.79 \times 10^{-4}$ in the *POL3* strain and $24.5 \pm 6.6 \times 10^{-4}$ in the *pol3-t* strain. After 24 hr at 25° in G_0/G_1, the frequency was $2.3 \pm 0.85 \times 10^{-4}$ in the *POL3* strain and $16.51 \pm 8.27 \times 10^{-4}$ in the *pol3-t* strain, and after 24 hr in G_0/G_1 at 30°, the frequency of intrachromosomal recombination was $1.79 \pm 0.77 \times 10^{-4}$ in the *POL3* strain and $21.69 \pm 2.8 \times 10^{-4}$ in the *pol3-t* strain. Thus, intrachromosomal recombination frequencies did not change after 24 hr of postincubation at 30° as compared to 25° in the absence of cell divisions. In contrast, recombination frequencies of *pol3-t* cells grown in parallel at 30° for the same amount of time were at least 2.5-fold higher than those at 25°, similar to the data in Table 2. Thus, DNA replication is most likely required for the development of the *pol3-t* hyperrecombination phenotype.

Effect of mutations in *rad1* and *rad52* on the *pol3-t* hyperrecombination phenotype: The excision repair gene *RAD1* is involved in intrachromosomal recombination (Klein 1988; Schiestl and Prakash 1988) and affects DNA DSB-induced recombination (Ivanov and Haber 1995). To study the effect of *RAD1* on the *pol3-t* hyperrecombination phenotype, we constructed strain AGY31 containing the *pol3-t* mutation and the *rad1* deletion. Single colonies of AGY30 and YR1-16 were incubated at 25° or 30°. Cells were counted and plated as described. In strain YR1-16 *POL3* wild type, the *rad1* deletion decreased intrachromosomal recombination frequencies 6- to 11-fold (Table 4). In strain AGY31 *rad1*Δ, the *pol3-t* mutation did not significantly increase intrachromosomal recombination at 25°, while at 30° intrachromosomal recombination increased 6.5-fold (Table 4). Thus, the *rad1* mutation decreased the *pol3-t*-mediated hyperrecombination phenotype 100-fold at 25° and 36-fold at 30°. Similar results were obtained with TCY1, TCY2, TCY3, and TCY4, another set of wild-type, *pol3-t*, *rad1*, and *rad1 pol3-t* strains (data not shown). Thus, the *pol3-t* hyperrecombination phenotype in most part is dependent on Rad1p.

Intrachromosomal recombination is also dependent

TABLE 3

Effect of *pol3-t* on reverse and forward mutation

	Mutation frequency ($\times 10^{-8}$)			
	RSY6 (*POL3*)		AGY30 (*pol3-t*)	
Mutation event	25°	30°	25°	30°
ilv1-92 to *ILV1*	147 ± 16	136 ± 17	904 ± 161***	1120 ± 353***
arg4-3 to *ARG4*	1.7 ± 0.9	1.5 ± 0.3	2.5 ± 0.2	4.8 ± 0.2***
CAN1 to *can1*^{R}	20 ± 7	15 ± 2	357 ± 183**	481 ± 267**

Results are reported as the mean of five or more independent experiments ± standard deviation. The probabilities refer to the comparison between the *POL3* strain and the *pol3-t* mutant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

TABLE 4

Effect of *RAD1* and *RAD52* on the hyperrecombination phenotype of *pol3-t*

Strain	Genotype	Intrachromosomal recombination ($\times 10^{-4}$)	
		25°	30°
RSY6	*RAD1 RAD52 POL3*	2.1 ± 1.4	3.5 ± 0.87
AGY30	*RAD1 RAD52 pol3-t*	32 ± 16**	76 ± 14***
YR1-16	*rad1Δ RAD52 POL3*	0.34 ± 0.01	0.32 ± 0.05
AGY31	*rad1Δ RAD52 pol3-t*	0.31 ± 0.22	2.1 ± 0.85***
YR52-2	*RAD1 rad52Δ, POL3*	0.19 ± 0.02	0.23 ± 0.06
AGY32	*RAD1 rad52Δ, pol3-t*	3.6 ± 0.52***	14 ± 1.0***
YR1-18	*rad1Δ, rad52Δ, POL3*	0.02 ± 0.01	0.03 ± 0.004
AGY33	*rad1Δ, rad52Δ, pol3-t*	0.13 ± 0.04**	1.7 ± 0.74**

Results are reported as the mean of five or more independent experiments ± standard deviation. All strains are isogenic. The probabilities refer to the comparison between the *POL3* strain and the *pol3-t* mutant in the different backgrounds. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

on the *RAD52* gene function (Schiestl and Prakash 1988; Klein 1995; Ivanov *et al.* 1996). The deletion of *rad52* in the *POL3* strain decreased the intrachromosomal recombination frequency 11- to 15-fold (Table 4). In the *rad52Δ pol3-t* strain AGY32, intrachromosomal recombination frequencies are 11- and 60-fold higher than those in the *rad52 POL3* strain YR52-2 at 25° and 30° (Table 4). Compared to AGY30, the *RAD52* wild-type *pol3-t* strain, the *rad52Δ* mutation decreased the intrachromosomal recombination frequency 9- and 5-fold at 25° and 30° (Table 4). Thus, the *pol3-t* hyperrecombination phenotype is partially dependent on Rad52p. In summary, the dependence of the *pol3-t* hyperrecombination phenotype on Rad1p is much greater than that on Rad52p.

The simultaneous deletion of the *rad1* and *rad52* genes led to a synergistic decrease in intrachromosomal recombination >100-fold in the YR1-18 *POL3* strain (Table 4), which has been seen before (Schiestl and Prakash 1988). In the AGY33 strain, which is *rad1Δ rad52Δ pol3-t*, intrachromosomal recombination frequencies are 6.5- and 57-fold higher than those in YR1-18 at 25° and 30° (Table 4). Thus, the *rad1* mutation, together with the *rad52* mutation, decreases the *pol3-t*-mediated hyperrecombination phenotype 246-fold and 45-fold at 25° and 30° (Table 4). However, this is due mostly to the *rad1* mutations since the *rad1Δ rad52Δ pol3-t* mutant showed a frequency similar to that of the *rad1Δ pol3-t* mutant. In summary, the *pol3-t* mutation still elevates the recombination frequency in the *rad1Δ rad52Δ* mutant background.

Effect of *pol3-t* on UV-induced intrachromosomal recombination: Some alleles of *POL3* are deficient in DNA-damage-induced mutagenesis and interchromosomal recombination (Giot *et al.* 1997). Thus, we determined the effects of *pol3-t* on intrachromosomal recombination induced by UV, γ-rays, and MMS and the effects of mutations *rad1* and *rad52* and of the double mutation *rad1 rad52* on DNA-damage-induced recombination events. Single colonies of these strains were incubated first at 25° and then for 4 hr at 30° and irradiated with UV and γ-rays or exposed to MMS as described in materials and methods.

UV irradiation induced a significant increase in intrachromosomal recombination at doses of 100 and 500 J/m^2 in both the wild-type (RSY6) and the *pol3-t* (AGY30) strains (Table 5). At 500 J/m^2, intrachromosomal recombination increased 4.5-fold ($P < 0.01$) in the wild type and 1.6-fold ($P < 0.05$) in the *pol3-t* strain (Table 5). Since the spontaneous frequency differs greatly between the two strains, it seems justified to also base the comparison on the average number of additional recombinants due to the radiation effect. At 500 J/m^2, there was an average of 22 added events in the wild type compared to an average of 8 added events in the *pol3-t* strain. This indicates that the *pol3-t* strain shows a mild deficiency in UV-induced intrachromosomal recombination.

The *pol3-t* mutation did not lower the survival after UV exposure of the *RAD* wild type and the *rad1Δ*, *rad52Δ*, and *rad1Δ rad52Δ* strains, suggesting that in these mutants UV-induced DNA-damage repair in the *pol3-t* mutant is as efficient as in the wild type (Table 5). If anything, the *pol3-t* mutant by itself, as well as in any of the double- and triple-mutant combinations, is slightly more UV resistant compared to the *POL3* genotype. The *rad1* mutant shows a dose-dependent, significant UV induction of intrachromosomal recombination starting at doses as low as 1 J/m^2, regardless of the *POL3* genotype. This dose is 100-fold lower than the dose resulting in significant induction in the *RAD* wild type. The *pol3-t* mutant still shows some minor defect in induced recombination since the dose of 20 J/m^2 resulted in an average of 9 added recombination events in the *rad1* mutant *vs.* an average of 3.7 added events in the *rad1 pol3-t* mutant (Table 5).

The *rad52* mutant as well as the *rad1 rad52* mutant were both completely defective in UV-induced intrachromosomal recombination. Both the *RAD1* and the *RAD52* pathways are involved in spontaneous recombination and the double mutant showed a synergistic de-

TABLE 5

Effect of *pol3-t* on UV-induced intrachromosomal recombination in *RAD*+, *rad1Δ*, *rad52Δ*, and *rad1Δ rad52Δ* strains

Strain	UV (J/m^2)	% survival	Deletion recombination ($\times 10^{-4}$)
RSY6 (*POL3*)	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 ± 6.0*
	500	39 ± 3.5	22 ± 3.0*
RSY6 *rad1Δ*	0	100	0.66 ± 0.22
	1	52 ± 12	2.9 ± 0.51***
	2	24 ± 13	5.5 ± 1.5***
	10	17 ± 2.5	4.7 ± 0.21***
	20	8.5 ± 2.5	9.7 ± 1.2***
RSY6 *rad1Δ pol3-t*	0	100	1.2 ± 0.42
	1	75 ± 3.4	2.7 ± 0.88**
	2	66 ± 6.1	2.9 ± 0.73**
	10	27 ± 6.8	3.4 ± 0.35***
	20	18 ± 1.5	4.9 ± 0.38***
RSY6 *rad52Δ*	0	100	1.1 ± 0.15
	10	73 ± 8.5	1.1 ± 0.15
	100	37 ± 12	1.3 ± 0.54
	200	35 ± 15	0.78 ± 0.59
	500	16 ± 0.0	0.92 ± 0.47
RSY6 *rad52Δ pol3-t*	0	100	9.8 ± 1.4
	10	79 ± 11	11 ± 0.75
	100	76 ± 6.1	15 ± 2.7**
	200	66 ± 7.5	11 ± 1.3
	300	26 ± 4	20 ± 0.78**
RSY6 *rad1Δ rad52Δ*	0	100	0.1 ± 0.02
	1	52 ± 34	0.08 ± 0.04
	2	27 ± 11	0.06 ± 0.008
	10	5 ± 0.0	0.08 ± 0.0
	20	4.4 ± 0.0	0.08 ± 0.007
RSY6 *rad1Δ rad52Δ pol3-t*	0	100	0.49 ± 0.3
	1	39.5 ± 4.5	0.36 ± 0.09
	2	27.5 ± 6.4	0.56 ± 0.04
	10	9.8 ± 0.2	1.3 ± 0.11***
	20	9 ± 0.8	1.1 ± 0.06**

Results are reported as the mean of five or more independent experiments ± standard deviation. All strains are isogenic. The probabilities refer to the comparison between the exposure and the untreated control. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

crease in spontaneous recombination, as previously found (Schiestl and Prakash 1988). The present results indicate that only the Rad52p, but not the Rad1p, pathway is involved in UV-induced recombination. In the *rad52*, as well as the *rad1 rad52*, background the *pol3-t* mutation resulted in a UV-induced recombination increase of about twofold.

Effect of *pol3-t* on γ-ray-induced intrachromosomal recombination: Irradiation with γ-rays induced significant increases in intrachromosomal recombination at

TABLE 6

Effect of *pol3-t* on γ-ray-induced intrachromosomal recombination in *RAD*+, *rad1*Δ, *rad52*Δ, and *rad1*Δ *rad52*Δ strains

Strain	γ-Rays (Gy)	% survival	Deletion recombination ($\times 10^{-4}$)
RSY6 (*POL3*)	0	100	2.4 ± 0.1
	50	65 ± 25	6.2 ± 0.9**
	500	29 ± 4.6	18 ± 2.3***
	1000	5 ± 2.4	39 ± 7.1***
AGY30 (*pol3-t*)	0	100	8.3 ± 0.5
	50	60 ± 11	12 ± 1.5*
	500	14 ± 2.5	23 ± 6.5*
	1000	3.8 ± 2.3	31 ± 9.7*
RSY6 *rad1*Δ	0	100	0.5 ± 0.05
	50	64 ± 17	1.1 ± 0.51*
	500	7.9 ± 2.5	2.2 ± 0.17***
	1000	0.2 ± 0.004	16 ± 3.6***
RSY6 *rad1*Δ *pol3-t*	0	100	0.66 ± 0.07
	50	64 ± 6.3	0.89 ± 0.19*
	500	14 ± 2.5	3.49 ± 1.26**
	1000	3.8 ± 2.3	6.9 ± 0.7***
RSY6 *rad52*Δ	0	100	0.14 ± 0.02
	1	43 ± 4.5	0.18 ± 0.03
	10	19 ± 1.2	0.2 ± 0.07
	50	7.3 ± 4	0.35 ± 0.1**
RSY6 *rad52*Δ *pol3-t*	0	100	0.99 ± 0.41
	1	51 ± 8.2	1.4 ± 0.19
	10	32 ± 3.8	2.0 ± 0.26**
	50	13 ± 4.6	3.3 ± 1.02**
RSY6 *rad1*Δ *rad52*Δ	0	100	0.014 ± 0.003
	1	57 ± 16	0.014 ± 0.004
	10	36 ± 10	0.015 ± 0.003
	50	10 ± 5.7	0.036 ± 0.017
RSY6 *rad1*Δ *rad52*Δ *pol3-t*	0	100	0.10 ± 0.03
	1	51 ± 11	0.14 ± 0.04
	10	31 ± 9	0.21 ± 0.07*
	50	11 ± 4.4	0.42 ± 0.05***

See legend for Table 5.

all doses used in both wild-type and *pol3-t* strains (Table 6). At 1000 Gy, intrachromosomal recombination increased 16-fold ($P < 0.001$) in the wild type and 3.7-fold ($P < 0.05$) in the *pol3-t* mutant (Table 6). At that dose there was an average of 37 added events in the wild type and an average of 23 added events in the *pol3-t* mutant, which is somewhat less in the mutant but rather similar.

In the *rad1*Δ strain, γ-ray exposure elevated recombination frequencies in a dose-dependent manner without any effect of *pol3-t*, while exposure resulted in a very moderate increase only at the highest dose in the *rad52*Δ and no significant induction in the *rad1*Δ *rad52*Δ strain (Table 6). Interestingly, as for UV-induced recombination in both backgrounds, in the *pol3-t* mutant there was significant induction at higher doses. In the *rad52*Δ *pol3-t* mutant, there was an average of 2.3 added events at a dose of 50 Gy *vs.* an average of 0.2 added events in the *rad52* mutant. Similarly, in the *rad1*Δ *rad52*Δ *pol3-t* mutant at the same dose, there was an average of 0.32 added events *vs.* an average of 0.022 added events in the *rad1*Δ *rad52*Δ strain.

Effect of *pol3-t* on MMS-induced intrachromosomal recombination: At higher doses of MMS the *pol3-t* mutation in the *RAD* wild type, the *rad1*Δ, and, to a lesser extent, the *rad52*Δ backgrounds were much more sensitive, which is in agreement with Blank *et al.* (1994;

TABLE 7

Effect of *pol3-t* on MMS-induced intrachromosomal recombination in *RAD*⁺, *rad1*Δ, *rad52*Δ, and *rad1*Δ *rad52*Δ strains

Strain	MMS (μg/ml)	% survival	Deletion recombination (×10⁻⁴)
RSY6 (*POL3*)	0	100	4.3 ± 1.0
	10	89 ± 15	9.3 ± 1.5**
	100	92 ± 8	39 ± 6.6***
	200	89 ± 15	51 ± 14**
	500	48 ± 19	102 ± 25***
AGY30 (*pol3-t*)	0	100	17 ± 4.6
	10	100	14 ± 8.4
	100	90 ± 0.5	30 ± 4.9*
	200	45 ± 1.5	60 ± 28
	500	0.8 ± 0.6	117 ± 2.8***
RSY6 *rad1*Δ	0	100	0.43 ± 0.07
	10	89 ± 11	1.9 ± 0.22***
	100	86 ± 14	8.0 ± 1.4***
	200	36 ± 8.5	9.5 ± 2.5***
	500	0.85 ± 0.2	13 ± 3.9***
RSY6 *rad1*Δ *pol3-t*	0	100	0.77 ± 0.31
	10	67 ± 27	3.4 ± 1.8*
	100	33 ± 2.5	6.4 ± 0.09***
	200	6.2 ± 3.7	14 ± 9.0***
	500	0.1 ± 0.04	30 ± 0.01***
RSY6 *rad52*Δ	0	100	0.30 ± 0.04
	10	71 ± 29	0.53 ± 0.10**
	50	54 ± 12	0.82 ± 0.01***
	100	8.5 ± 1.5	1.4 ± 0.62**
	200	0.23 ± 0.09	3.1 ± 3.9***
RSY6 *rad52*Δ *pol3-t*	0	100	7.3 ± 2.6
	10	59 ± 26	25 ± 3.42***
	50	8 ± 1.5	30 ± 2.1***
	100	2 ± 0.2	35 ± 3.1***
	200	0.1 ± 0.03	34 ± 5.7***
RSY6 *rad1*Δ *rad52*Δ	0	100	0.01 ± 0.00
	1	39 ± 9	0.06 ± 0.03**
	10	33 ± 5	0.32 ± 0.22*
	50	13 ± 3.5	0.19 ± 0.1**
	100	0.9 ± 0.0	0.25 ± 0.001***
RSY6 *rad1*Δ *rad52*Δ *pol3-t*	0	100	0.43 ± 0.16
	1	99 ± 0.5	0.51 ± 0.11
	10	89 ± 0.5	0.97 ± 0.26**
	50	4.2 ± 0.5	2.3 ± 0.05***
	100	1.5 ± 0.03	5.3 ± 2.4**

See legend for Table 5.

Table 7). Since the different *rad* mutant backgrounds caused MMS sensitivity to different degrees, one way to compare the *pol3-t* effect is to compare sensitivities at a dose giving ∼40–50% viability in the *POL3* wild-type strain. In the *RAD* wild-type strain, exposure to 500 μg/ml MMS resulted in 48% viability with a 60-fold lower viability in the *pol3-t* strain. In comparison, 200 μg/ml MMS exposure in the *rad1*Δ strain had a viability of 36% compared to a 6-fold lower viability in the *rad1*Δ *pol3-t* strain. At a dose of 50 μg/ml MMS, the *rad52*Δ strain had a viability of 54% compared to a 7-fold lower viability in the *rad52*Δ *pol3-t* strain. Finally, at a dose of 1 μg/

TABLE 8

Effect of RAD1, RAD52 deletion on the *pol3-t* temperature-sensitive phenotype

	% survivors			
Time (hr)	*pol3-t* RAD	*pol3-t rad1*	*pol3-t rad52*	*pol3-t rad1 rad52*
0	100	100	100	100
1	71.6 ± 15.1	65.5 ± 9.7	50.6 ± 2.5	51.4 ± 2.5
2	62.2 ± 10.0	36.9 ± 4.9*	31.4 ± 1.0**	33.5 ± 1.9**
4	29.9 ± 4.9	11.8 ± 1.2**	7.1 ± 1.9**	11.2 ± 0.9**
6	3.4 ± 1.5	1.1 ± 0.7	0.5 ± 0.07*	0.9 ± 0.2*
8	0.4 ± 0.2	0.05 ± 0.02*	0.15 ± 0.03*	0.09 ± 0.03*

Single colonies were inoculated in 5 ml YPAD and grown at 25° for 17 hr. Cells were washed, resuspended in 5 ml prewarmed (37°) YPAD at the cell concentration of 3×10^7/ml, and incubated at 37°. At time points 0, 1, 2, 4, 6, and 8 hr after the incubation, cells were counted and plated in YPAD to score survivors. For better comparison, the viability at time point 0 was set at 100%. The actual viability percentages at the 0 time point were not <95% for any of the strains. Colonies were counted after 3 days at 25°. Cell counts went down no more than 15% compared to those at time point 0. After 1 hr at 37°, cells were arrested as small budded cells, and then cells elongated.The probabilities refer to the comparison between the *pol3-t* RAD^+ strain and the different *rad* mutants. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

ml MMS, the *rad1*Δ *rad52*Δ strain had a viability of 39% compared to an ~3-fold higher viability in the *rad1*Δ *rad52*Δ *pol3-t* strain. Thus, the *pol3-t*-mediated MMS sensitivity was diminished in both the *rad1* and the *rad52* background and absent in the *rad1 rad52* background.

At the lowest dose of MMS (10 μg/ml), intrachromosomal recombination increased 2-fold ($P < 0.01$) in the wild type while no increase was seen in the *pol3-t* mutant (Table 7). At equitoxic doses at the same survival level of 40–50%, at a dose of 500 μg/ml in the wild type and of 200 μg/ml in the *pol3-t* mutant, MMS increased intrachromosomal recombination 24-fold ($P < 0.001$) in the wild type and 3.5-fold (nonsignificant) in the *pol3-t* mutant. At these doses, there was an average of 98 added events in the wild type and an average of 43 added events in the *pol3-t* mutant. This would allow the conclusion that for all three DNA-damaging agents, the results indicate that the level of DNA-damage-induced recombination is lower in the *pol3-t* mutant than in the *RAD* wild type, indicating that Pol3p is partially responsible for DNA-damage-induced intrachromosomal recombination. If, however, the same dose rather than equitoxic doses of MMS is evaluated, there is an equal level of induction of intrachromosomal recombination in the *pol3-t* mutant.

In the *rad1*Δ, *rad52*Δ, and *rad1*Δ *rad52*Δ double-mutant background, MMS induced recombination at all doses regardless of the *POL3* status (Table 7). However, again as for UV- and γ-ray-induced recombination, in both backgrounds the *pol3-t* mutant showed higher inducibility. At a dose of 100 μg/ml MMS, there was an average of 27.7 added events in the *rad52*Δ *pol3-t* strain compared to an average of 1.1 added events in the *rad52*Δ strain. At the same dose in the *rad1*Δ *rad52*Δ *pol3-t* strain, there were ~5 induced events compared to 0.24 induced events for the *rad1*Δ *rad52*Δ strain (Table 7).

Effect of *rad1* and *rad52* mutations on the temperature-sensitive phenotype of *pol3-t*: Since the *rad1* and *rad52* mutations partially reduced the hyperrecombination phenotype of the *pol3-t* mutant, we determined the effect of mutations in these DNA repair pathways on the temperature-sensitive phenotype of *pol3-t*. The *pol3-t* mutant and all combinations of double and triple mutants were incubated at the restrictive temperature, and viability was determined after different time points up to 8 hr. After 1 hr, the cells were already arrested and did not grow further. The control *POL3 rad* mutants were incubated in the same way but kept dividing rapidly at 37°. Thus, the viability must have been high in these strains. After 8 hr, the *pol3-t* mutant had 0.4% viable cells. At all time points the survival of the double and triple mutants was lower than that of the *pol3-t* single mutant and at almost all time points starting at the 2-hr point this difference was significant (Table 8). This indicates that both the Rad1p and the Rad52p pathway are partially involved in the repair of lethal DNA lesions in the *pol3-t* strain at the restrictive temperature.

Effect of *pol3-t* heterozygosity on recombination and MMS sensitivity: A decrease in the expression of *POL3* under the *GAL1* promoter is sufficient to cause a mutator phenotype and MMS sensitivity (Kokoska *et al.* 2000). Thus, we determined whether a *pol3-t/POL3* heterozygous strain showed any effect on recombination efficiency or on MMS sensitivity at the restrictive temperature of 37°. There was a significant difference for both intrachromosomal and interchromosomal recombination at the restrictive temperature in the *pol3-t/POL3* heterozygous mutant (Table 9). The heterozygous strain was also more MMS sensitive at every dose and, starting

TABLE 9

Effect of *pol3-t/POL3* heterozygosity on recombination and MMS sensitivity

Strain	HIS3 recombinants[a] ($HIS3^+/10^4$ cells)	ADE2 recombinants[a] ($ADE2^+/10^5$ cells)	MMS[b] (mg/ml)	% survival[b]
RS112(*POL3/POL3*)	0.51 ± 0.23	0.38 ± 0.06	0	100
			0.05	60.3 ± 5.7
			0.1	20.6 ± 2.8
			0.2	15.5 ± 2.7
			0.4	2.1 ± 0.7
			0.8	0.15 ± 0.05
AGY37(*pol3-t/POL3*)	1.25 ± 0.40**	0.66 ± 0.18*	0	100
			0.05	55.7 ± 8.2
			0.1	17.6 ± 3.5
			0.2	10.2 ± 2.0*
			0.4	0.6 ± 0.08**
			0.8	0.07 ± 0.008***

The probabilities refer to the comparison between the *POL3/POL3* wild-type strain and the *pol3-t/POL3* heterozygous mutant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

[a] Three independent colonies of strains RS112 (*POL3/POL3*) and seven cultures of AGY37 (*pol3-t/POL3*) were grown at 37° for 17 hr and deletion and interchromosomal recombination frequencies were determined. This experiment was done in duplicate. RS112 cells grew on average for 4.8 generations and AGY37 for 4.0 generations.

[b] From overnight cultures grown in YPAD at 25°, 1×10^8 cells/ml were inoculated in 5 ml YPAD at 37° for 6 hr. Then cells were counted and 10^7 cells/ml were further inoculated in 5 ml prewarmed YPAD containing the indicated concentrations of MMS for 17 hr at 37°. Cells were washed, counted, plated onto YPAD plates, and incubated at 25° for 3 days. Each data point represents the mean ± standard deviation of four independent experiments.

at a dose of 0.2 mg/ml (Table 9), this increase was significant. Thus, the *pol3-t/POL3* heterozygous mutant at the restrictive temperature showed an increase in recombination frequency as well as an MMS-sensitive phenotype.

DISCUSSION

In this study, we found that the *pol3-t* allele of the *POL3/CDC2* gene of *S. cerevisiae*, which encodes the catalytic subunit of the DNA polymerase δ, increased intra- and interchromosomal recombination in a diploid and intrachromosomal recombination in two haploid strains. Previous studies reported that mutations in *POL3/CDC2* genes increased intrachromosomal recombination between homologous and homeologous DNA sequences (Aguilera and Klein 1988; Tran *et al.* 1997) and increased interchromosomal recombination (Hartwell and Smith 1985; Giot *et al.* 1997). The elevated frequency of recombination in the *pol3-t* mutant could be mechanistically similar to the events in the *POL3* wild type but just occur more frequently. Thus, we sought to further characterize the *pol3-t*-mediated hyperrecombination phenotype. The present study adds to the previously published findings by the characterization of effects of *rad1* and *rad52* mutations on the *pol3-t*-mediated hyperrecombination phenotype in isogenic backgrounds as well as the effects of exposure to UV, ionizing radiation, and MMS on deletion recombination in the different DNA repair mutants. In addition, we show that the *pol3-t*-mediated hyperrecombination phenotype is dependent on DNA replication and that even the *pol3-t/POL3* heterozygous mutation increased recombination and MMS sensitivity.

Moreover, we found that *pol3-t* increased both reverse and forward mutation frequencies. Most interestingly, the *pol3-01* mutant has abnormal cell-cycle progression due to activation of the S-phase checkpoint, and inactivation of the S-phase checkpoint suppressed the cell-cycle progression defect as well as the mutator phenotype (Datta *et al.* 2000). This indicates that activation of the checkpoint might have resulted in the accumulation of the mutations.

The *pol3-t*-mediated hyperrecombination phenotype requires DNA replication: To determine whether cell division and/or DNA replication affect the hyperrecombination phenotype, we monitored intrachromosomal recombination during a prolonged G_1 arrest. The frequency of intrachromosomal recombination did not change during the cell-cycle arrest and did not increase in G_1-arrested cells at 30°. Thus, cell division or DNA replication is necessary to increase recombination in *pol3-t* strains.

Intrachromosomal recombination events leading to

deletions are due mainly to DNA DSBs (Galli and Schiestl 1995, 1998b). Single-strand breaks in the region between the *HIS3* duplication or exposure to alkylating agents or UV light did not increase deletion recombination unless DNA replication occurred (Galli and Schiestl 1998b, 1999). Thus, single-strand breaks may be converted into DNA DSBs by DNA replication on a single-strand or chemically damaged DNA template (Kaufmann and Paules 1996; Galli and Schiestl 1998b). These DSBs could induce SSA or one-sided invasion.

The hyperrecombination phenotype in *pol3-t* strains depends partially on Rad52p but much more so on Rad1p: Intrachromosomal recombination events leading to deletions between repeated sequences can occur by several mechanisms: by recombination between the two repeats within one chromatid as intrachromatid exchange; by SSA; by one-sided invasion events; or, alternatively, by recombination between sister chromatids as unequal sister chromatid exchange or sister chromatid conversion (Schiestl *et al.* 1988; Haber 1992; Belmaaza and Chartrand 1994; Galli and Schiestl 1995, 1998b; Klein 1995). Previous studies on cell-cycle-arrested cells suggested that SSA and/or one-sided invasion are the preferential mechanisms by which such deletions occur (Galli and Schiestl 1998b).

Deletion of *RAD1* and *RAD52* greatly reduces the frequencies of intrachromosomal deletion events between repeats (Schiestl and Prakash 1988; Thomas and Rothstein 1989; Liefshitz *et al.* 1995; Saparbaev *et al.* 1996). Different types of intrachromosomal recombination events are controlled by Rad1p and Rad52p. Deletion events occurring by SSA events are dependent on the Rad1p function (Fishman-Lobell and Haber 1992; Prado and Aguilera 1995; Paques and Haber 1999) whereas conversion events or deletion events occurring by one-sided invasion are probably Rad52p dependent (Klein 1988; Schiestl and Prakash 1988; Prado and Aguilera 1995).

SSA, one of the mechanisms for the deletion recombination events, requires Rad1p and Rad10p if the distance between interacting repeats is >60 bp (Paques and Haber 1999). It has been argued that deletions <60 bp may be due to polymerase slippage (Kokoska *et al.* 2000). It seems, however, inconceivable that slippage could occur over a distance of 6 kb in the absence of inverted repeats as would be required for the deletions in our construct. Thus, it is more likely that the events happen by SSA or by one-sided invasion. DNA DSBs could be generated by replication of a single-strand break or single-strand interruption that would be expected to be more prevalent or longer lasting in the *pol3-t* mutant. Such DSBs could initiate Rad1p-dependent SSA events. Since most *pol3-t*-induced recombination events are Rad1p mediated, this pathway could account for the majority of the events. On the other hand, long stretches of single-strand DNA on the lagging-strand template in the *pol3-t* mutant (Gordenin *et al.* 1992; Kokoska *et al.* 1998) could potentially invade the second copy of the *HIS3* duplication in our recombination substrate, leading to Rad52p-dependent, one-sided invasion-like events.

The low level of recombination went up significantly in the *rad52* mutant in the presence of the *pol3-t* mutation. In addition, ionizing radiation induced recombination to much higher levels in the *pol3-t rad52* double mutant than in the *rad52* single mutant. This indicates that *pol3-t* channels lesions into a Rad52p-independent pathway, like the Rad1p pathway.

Most of the *pol3-t*-induced recombination events were dependent on Rad1p or Rad52p; however, the *pol3-t* mutation still increased the frequency in the absence of Rad1p and Rad52p. Other hyperrecombination phenotypes differ in their dependence on the Rad1p- and/or Rad52p-mediated pathways. A mutation in *hpr1* increases recombination between DNA repeats up to 2000-fold (Aguilera and Klein 1988; Santos-Rosa and Aguilera 1994). Since *hpr1* mutants also show 100-fold elevated frequencies of chromosome loss, it has been suggested that the hyperrecombination phenotype is due to DNA breaks (Santos-Rosa and Aguilera 1994). This increased recombination frequency is completely abolished in a *rad1 rad52* double-mutant background (Santos-Rosa and Aguilera 1994), which makes it different from the *pol3-t* effect. DNA transcription also induces recombination (Voelkel-Meiman *et al.* 1987; Thomas and Rothstein 1989). Both systems of transcription stimulated recombination. In the *GAL10* assay as well as in the *HOT1* assay, most events are Rad52p dependent and fewer events Rad1p dependent (Thomas and Rothstein 1989; Zehfus *et al.* 1990), which makes it different from the *pol3-t* genetic control. In both assays, a small proportion of the transcription-induced recombination events are Rad1p Rad52p independent (Thomas and Rothstein 1989; Zehfus *et al.* 1990). Among mutations isolated in a screen for an increase in recombination in a *rad1*Δ *rad52*Δ double-deletion mutant strain, the *rfa1-D228Y* mutant has been found to stimulate intrachromosomal deletion events between repeats up to the wild-type level (Smith and Rothstein 1995). The hyperrecombination phenotype of the *rfa* mutation is much more dependent on Rad1p than on Rad52p and, out of several possible recombination events between repeats, DNA deletions display the greatest stimulation in the *rad1 rad52* double-mutant background (Smith and Rothstein 1999). Thus, the authors proposed that the *rfa*-mediated hyperrecombination phenotype is most likely caused by a Rad1p Rad52p-independent SSA mechanism (Smith and Rothstein 1999). In a similar way, the weak hyperrecombination phenotype we observed in the *rad1*Δ *rad52*Δ *pol3-t* strain may occur by a Rad1p Rad52p-independent SSA mechanism.

Genetic control of DNA-damage-induced intrachromosomal recombination in strains of different *POL3* status: The *pol3-t* mutant was slightly more UV resistant in all genotypes compared to the *POL3* wild type. This might be due to the somewhat longer time available to repair the lesions by excision repair in the *pol3-t* mutant since the cells grew at 30° for 4 hr prior to UV exposure. There was no such difference in survival for γ-rays or MMS. The *pol3-t* mutant was MMS sensitive as previously reported for other *pol3* mutants (Blank *et al.* 1994). This MMS sensitivity was diminished in both the *rad1* and the *rad52* background and absent in the *rad1 rad52* double mutant, which may indicate that Pol3p may be important for both the excision repair and the recombination repair pathway of MMS repair.

The *pol3-t* strain was partially defective in UV- and γ-ray-induced intrachromosomal recombination in agreement with findings by others (Fabre *et al.* 1991; Giot *et al.* 1997). DNA polymerase δ is required for both DNA replication and base excision repair (Budd and Campbell 1993, 1995; Blank *et al.* 1994; Morrison and Sugino 1994). Recently, a new role of the DNA polymerase δ in the DNA DSB repair and DSB-induced mitotic gene conversion has been reported (Holmes and Haber 1999). According to all models of recombination (Orr-Weaver and Szostak 1985), DNA replication is involved in processes such as extension of strand displacement and repair of gaps.

DNA-damage-induced intrachromosomal deletion recombination events are under different genetic control than spontaneous events, suggesting a difference in mechanism. UV and γ-rays induced recombination in the *rad1*Δ strain, but not at all or very little in the *rad52*Δ and the *rad1*Δ *rad52*Δ strains. This demonstrated that UV- and γ-ray-induced intrachromosomal recombination required Rad52p but not Rad1p in a *POL3* background, whereas spontaneous recombination is dependent on both Rad1p and Rad52p functions (Schiestl and Prakash 1988; Thomas and Rothstein 1989; Liefshitz *et al.* 1995; Saparbaev *et al.* 1996). Thus, it is possible that different pathways of recombination might be preferred in spontaneous *vs.* DNA-damage-induced recombination. Interestingly, in the *pol3-t* background, UV- and γ-ray-induced intrachromosomal recombination was Rad52p independent. Both pathways of hyperrecombination potentially operable in the *pol3-t* strain, involving DSB formation on a single-strand break or gap template as well as invasion of homologous DNA by long stretches of single-strand DNA on the lagging-strand template, could be more prevalent after additional DNA damage. As much of the *pol3-t* hyperrecombination pathway was independent of Rad52p so was the UV- or γ-ray-induced recombination in the *pol3-t* mutant.

***Pol3-t* heterozygosity results in hyperrecombination and MMS-sensitive phenotypes:** Our data indicate that *pol3-t/POL3* heterozygosity significantly increased the recombination frequency in both systems as well as the MMS sensitivity at the restrictive temperature. This could be due to the fact that the *pol3-t* allele may have some dominant effect, such as binding to a multi-enzyme complex as an inactive component, or that just a lower level of Pol3p leads to the recombinagenic effect. Since a lower level of Pol3p obtained by repressing the gene under the *GAL1* promoter resulted in a mutator as well as in an MMS-sensitive phenotype (Kokoska *et al.* 2000), it is likely that a lower amount of Pol3p in the heterozygous mutant at the restrictive temperature is responsible for the effect in our experiment. In agreement with our finding, it has also been shown that the homozygous as well as the heterozygous *pol3-01* mutation is inviable in combination with a mutation in *RAD27* (Gary *et al.* 1999). Our results indicate that a mutation in DNA polymerase δ even in a heterozygous combination might increase the frequency of genetic instability, which might be a risk factor for cancer.

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