

Involvement of the Yeast DNA Polymerase δ in DNA Repair *in Vivo*

Loïc Giot¹, Roland Chanet, Michel Simon, Céline Facca and Gérard Faye

Institut Curie-Biologie, Centre Universitaire, 91405 Orsay Cédex, France

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ABSTRACT

The *POL3* encoded catalytic subunit of DNA polymerase δ possesses a highly conserved C-terminal cysteine-rich domain in *Saccharomyces cerevisiae*. Mutations in some of its cysteine codons display a lethal phenotype, which demonstrates an essential function of this domain. The thermosensitive mutant *pol3-13*, in which a serine replaces a cysteine of this domain, exhibits a range of defects in DNA repair, such as hypersensitivity to different DNA-damaging agents and deficiency for induced mutagenesis and for recombination. These phenotypes are observed at 24°, a temperature at which DNA replication is almost normal; this differentiates the functions of *POL3* in DNA repair and DNA replication. Since spontaneous mutagenesis and spontaneous recombination are efficient in *pol3-13*, we propose that *POL3* plays an important role in DNA repair after irradiation, particularly in the error-prone and recombinational pathways. Extragenic suppressors of *pol3-13* are allelic to *sdp5-1*, previously identified as an extragenic suppressor of *pol3-11*. *SDP5*, which is identical to *HYS2*, encodes a protein homologous to the p50 subunit of bovine and human DNA polymerase δ . *SDP5* is most probably the p55 subunit of Pol δ of *S. cerevisiae* and seems to be associated with the catalytic subunit for both DNA replication and DNA repair.

BOTH prokaryotic and eukaryotic cells repair DNA damage through three main pathways (base or nucleotide excision repair, error-prone repair and recombinational repair) requiring DNA synthesis. In *Escherichia coli*, DNA repair synthesis is carried out by DNA polymerase I, which also catalyzes gap-filling synthesis, joining Okazaki fragments on the lagging strand during DNA replication. DNA polymerase III holoenzyme catalyzes highly processive synthesis on both strands during DNA replication (KORNBERG and BAKER 1992).

In eukaryotic cells, the situation is more complex, since three of five DNA polymerases, namely Pol α , Pol δ , and Pol ϵ , are required for DNA replication. In *Saccharomyces cerevisiae*, the genes encoding the catalytic subunit, *CDC17* (*POL1*) for Pol α , *CDC2* (*POL3*) for Pol δ and *POL2* for Pol ϵ , are essential (JOHNSON *et al.* 1985; BOULET *et al.* 1989; SITNEY *et al.* 1989; MORRISON *et al.* 1990). Pol α has an associated primase activity and is responsible for initiation on both leading and lagging strands (BROOKS and DUMAS 1989). Both Pol δ and Pol ϵ can extend the primers formed by Pol α (BURGERS 1991; PODUST and HUBSCHER 1993), though their specific assignment at the replication fork is not yet clear.

Concerning the respective involvement of Pol δ and Pol ϵ in DNA repair synthesis, ambiguity remains. Both DNA polymerases appear to play a role in base excision repair (WANG *et al.* 1993; BLANK *et al.* 1994) and nucleo-

tide excision repair (BUDD and CAMPBELL 1995). Pol δ seems to be required for induced gene conversion *in vivo* (FABRE *et al.* 1991), whereas Pol ϵ has been isolated as a part of a recombinational complex *in vitro* (JESSBERGER *et al.* 1993). Finally, both polymerase activities are enhanced by the proliferating cell nuclear antigen (PCNA) (NG *et al.* 1991), which is implicated in DNA repair of UV-induced lesions (SHIVJI *et al.* 1992).

The two nonessential DNA polymerases, Pol β (PRASAD *et al.* 1993) and Pol ζ (Rev3p) (MORRISON *et al.* 1989; NELSON *et al.* 1996), have been attributed to two different pathways; Pol β is possibly involved in double-strand break DNA repair (LEEM *et al.* 1994), while Pol ζ has been proposed for the error-prone repair response to DNA damage (MORRISON *et al.* 1989).

The central part of the sequence of each catalytic subunit of Pol α , δ and ϵ contains conserved motifs that define the regions performing DNA polymerization (BRAITHWAITE and ITO 1993). The N-terminal domains of both Pol δ and ϵ contain three conserved boxes involved in their exonuclease proofreading activity (SIMON *et al.* 1991; MORRISON *et al.* 1993). These sequences are not found in Pol α , which lacks this activity. The C-terminal domain of all these polymerases is cysteine rich. It has been recently proposed that the C-terminal domain of Pol ϵ acts as a sensor of DNA replication able to stimulate DNA repair in response to replication blocks (NAVAS *et al.* 1995). DNA polymerase δ from human to yeast displays a very conserved region surrounding the cysteine-rich domain (CULLMANN *et al.* 1993; and see Figure 1). To examine the function of the C-terminal domain of Pol δ , we performed several amino acids changes of the cysteine residues and ana-

Corresponding author: Gérard Faye, Institut Curie-Biologie, CNRS UMR216, Bat.110, Centre Universitaire, 91405 Orsay Cédex, France. E-mail: faye@curie.u-psud.fr

¹Present address: Department of Molecular Genetics and Microbiology, State University of New York at Stony Brook, Stony Brook, NY 11794-5222.

TABLE 1
Yeast strains

Strain	Genotype	Source
GF260-2	<i>MATa ura3 leu2 trp1 lys2</i>	SIMON <i>et al.</i> (1993)
GF262-2	<i>MATα ura3 leu2 trp1 his3</i>	SIMON <i>et al.</i> (1993)
DG146	GF260-2 × GF262-2	GIOT <i>et al.</i> (1995)
GL329	<i>MATa ura3 leu2 trp1 lys2 pol3-13</i>	This work
GL330	<i>MATα ura3 leu2 trp1 his3 pol3-13</i>	This work
GL355	<i>MATa ura3 leu2 trp1 lys2 pol3-13 sdp5-15</i>	This work
GL356	<i>MATα ura3 leu2 trp1 his3 pol3-13 sdp5-15</i>	This work
GF289 (501)	<i>MATa ura3 leu2 trp1 lys2 cyh2^R pol3::TRP1-CYH2 (pGL330)</i>	SIMON <i>et al.</i> (1993)
GF279-1C	<i>MATα ura3 leu2 trp1 lys2 cyh2^R</i>	This work
GF279-1D	<i>MATa ura3 leu2 trp1 his3 cyh2^R</i>	This work
DG178-1	GF279-1C × GF279-1D	This work
GF300-2B	<i>MATa ura3 leu2 trp1 his3 pol3-11</i>	This work
GF300-5D	<i>MATa ura3 leu2 trp1 lys2 pol3-11</i>	This work
GF1401	<i>MATa ura3 leu2 trp1 lys2 cyh2^R pol3-11</i>	This work
GF1572	<i>MATa ura3 leu2 trp1 lys2 cyh2^R pol3-11 sdp5-1</i>	This work
DG177-1	GF356 × GL329	This work
GF308-2C	<i>MATa ura3 leu2 trp1 lys2 sdp5-15</i>	This work
GF318-2C	<i>MATa ura3 leu2 trp1 his3 pol3-14</i>	This work
GF326-9-1	<i>MATa ura3 leu2 trp1 his3 pol3-14 SDP5-URA3^a</i>	This work
GF263-5B	<i>MATα ura3 leu2 trp1 lys2</i>	This work
GF327-1C	<i>MATa ura3 leu2 trp1 lys2 SDP5-URA3^a</i>	This work
GF305-3B	<i>MATα ura3 leu2 trp1 his3 pol3-11 sdp5-1</i>	This work
GF324-2A	<i>MATα ura3 leu2 trp1 lys2 pol3-14 sdp1</i>	This work
AA1028	<i>MATα ura3 leu2 trp1 arg4B</i>	A. ADJIRI
AA1026	<i>MATa ura3 leu2 trp1 arg4R</i>	A. ADJIRI
FF181134	<i>MATa ura3 leu2 trp1 his7 lys1 rev3::URA3</i>	F. FABRE
FF739	<i>MATa ura3 leu2 trp1 his7 lys1 rad18::LEU2</i>	F. FABRE
AA1987	<i>MATa ura3 leu2 trp1 arg4 rad1::LEU2</i>	F. FABRE
FF958	<i>MATa ura3 leu2 trp1 his7 lys1 rad51::URA3</i>	F. FABRE
FF742	<i>MATa ura3 leu2 trp1 his7 lys1 rad52::URA3</i>	F. FABRE
FF964	<i>MATa ura3 leu2 trp1 his7 lys1 rad50::URA3</i>	F. FABRE

Strains from GF260-2 to GF324-2A are isogenic.

^a *SDP5-URA3* means that *URA3* was inserted near the gene *SDP5*.

lyzed the phenotype of the resulting mutants. These phenotypes indicate that this domain is essential for DNA replication and also suggest the involvement of Pol δ in error-prone and recombination DNA repair.

MATERIALS AND METHODS

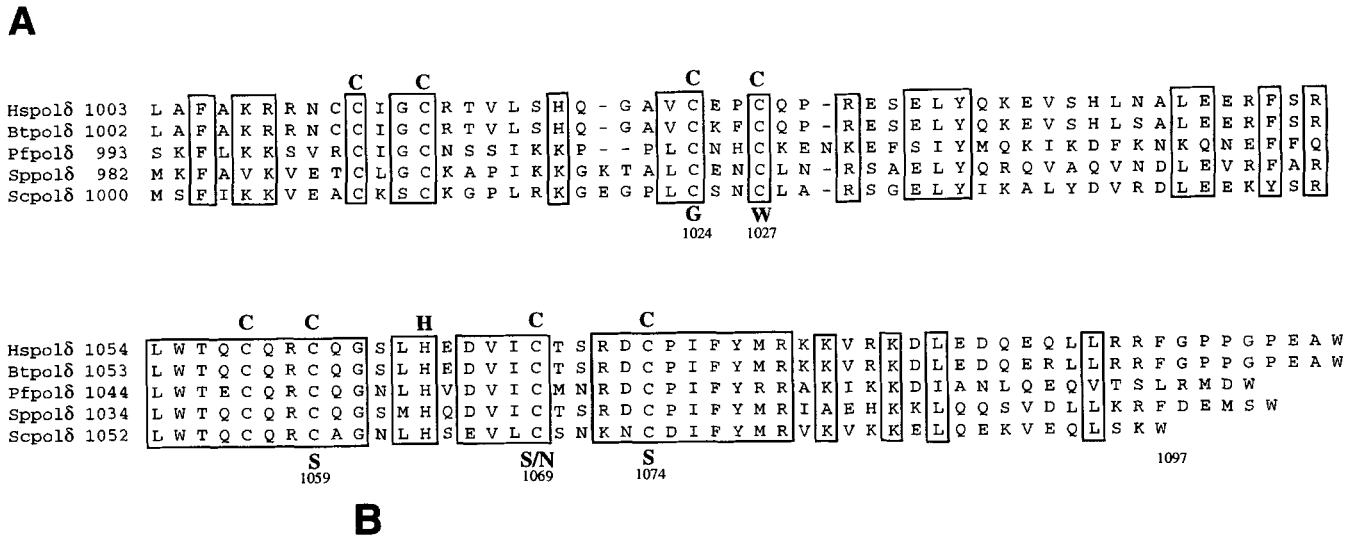
Strains and media: The yeast strains used are listed in Table 1. Yeast culture media were as described by SHERMAN (1991). All procedures for manipulating DNA rely on standard methods. The yeast lithium transformation procedure was performed according to SCHIESTL and GIETZ (1989).

Thermosensitivity was checked either by microscopic examination of complete medium liquid cultures for the presence of the characteristic dumbbell phenotype or by observation of the growth on solid medium at various temperatures (GIOT *et al.* 1995). Thermoinactivation was determined by plating known numbers of cells on complete solid medium, incubating them at restrictive temperature for given periods of times and then shifting them to the permissive temperature (24°) for 4 to 5 days.

Measurement of spontaneous mutation or recombination rates: Spontaneous mutation or recombination rates were measured as in LEA and COULSON (1949) and VON BORSTEL (1978), respectively; the experiments were done twice with

nine cultures for forward spontaneous mutation and four times with seven cultures for spontaneous intragenic recombination.

Site-specific mutagenesis: Single amino acid changes in the C-terminal part of *POL3* were constructed in pGR22Xho, which is derived from pGR22 (SIMON *et al.* 1993) by creating an *XhoI* site at position 3194 in *POL3* with oligonucleotide CCATTATTTGACTCGAGAACAATATT. The following oligonucleotides were used to create the indicated mutations: C1024G (GCCCTCTTGGTTCGAAGTGTCT); C1027W (TTC AAATTGGCTAGCAAGG); C1059S (TGCCAAAGGAGCGCT GGTA); C1069N (GAAGTTTTGAATTCAAATAA) and C1074S (AAATAAGAATTCTGACATTTT). The oligonucleotides were designed to generate a restriction site. Plasmids bearing mutations were screened by restriction analysis and the *XhoI-PstI* DNA fragments were sequenced and reintroduced into pGR22Xho. The pGR22Xho-derived plasmids were digested with *HindIII* and *SacI*. The digestion products were used to transform strains GF289(501), in which the deleted C-terminal part of gene *POL3* was complemented by the *URA3* centromeric plasmid YCp50 carrying a wild-type copy of *POL3* (plasmid pGL330) (SIMON *et al.* 1993). Ten *Cyh^R* and *Trp⁻* transformants were tested for their ability to grow on 5FOA plates (BOEKE *et al.* 1987), *i.e.*, for their propensity to lose the plasmid pGL330 carrying *POL3*. The mutants able to grow on this selective medium were checked by Southern



Modifications	Growth phenotype
C1024G	Wild type
C1027W	Lethal
C1059S	Wild type
C1069S*	Thermosensitive
C1069N	Lethal
C1074S	Thermosensitive

FIGURE 1.—Localization and phenotype of mutants in the cysteine-rich domain of Pol δ . (A) Amino acid sequence homology between the C-terminal ends of the catalytic DNA polymerase δ subunit of human (Hs pol δ ; CHUNG *et al.* 1991), *Bos taurus* (Bt pol δ ; ZHANG *et al.* 1991), *Plasmodium falciparum* (Pf pol δ ; RIDLEY *et al.* 1991), *Schizosaccharomyces pombe* (Sp pol δ ; PIGNÈDE *et al.* 1991) and *Saccharomyces cerevisiae* (Sc pol δ ; FAYE *et al.* 1992). Amino acid residues are boxed in the column when at least two residues belonging to Pf, Sp or Sc pol δ are identical to the corresponding residues of Hs or Bt pol δ . Conserved cysteines are written above the boxes. Substitutions of cysteine residues are indicated. (B) Phenotypes of the mutants. *This mutation was obtained in a previous work (GIOT *et al.* 1995).

blotting for the presence of the restriction site introduced with each mutation. Those showing a lethal or thermosensitive phenotype on 5FOA plates were crossed with strain GF262-2, the diploids were selected, replica-plated onto 5FOA and induced to sporulate, and tetrads were analyzed to follow the segregation of lethal or thermosensitive phenotypes. Southern blottings were carried out with the DNA of diploids to verify that they contained one wild-type and one mutated copy of gene *POL3*.

Cloning of *sdp5-15*: Total DNA from strain GL356 was partially digested with *Sau3A*. DNA fragments ranging from 6 to 9 kilobases (kb) were isolated. Plasmid p370 [YRP71, FAYE and SIMON 1982, containing the kanamycin Gen Block (Pharmacia) inserted at its unique *EcoRI* site] was cleaved with *XhoI* restriction enzyme. Before ligation, *Sau3A* and *XhoI* overhanging ends were partially filled with A+G and C+T, respectively, using Taq DNA polymerase (Promega) to prevent self-ligation (ROSE and BROACH 1991). To confirm the identity of the cloned gene, we introduced the *URA3* marker near the *SDP5* locus. To do so, the 1.9-kb *EcoRI-XbaI* DNA fragment located at the left end of gene *SDP5* was inserted between the *EcoRI/NheI* sites of Ylp5. This plasmid was integrated in strains GF318-2C by transformation and its integration was checked by Southern blotting. A *Ura*⁺ transformant, named GF326-9-1, was crossed with strain GF263-5B (*POL3*, *SDP5*). After sporulation, a *POL3 SDP5(URA3)* spore, named GF327-1C,

was isolated. Strain GF327-1C was crossed with GL356 (*pol3-13 sdp5-15*) or GF305-3B (*pol3-11 sdp5-1*) and segregation of thermosensitivity and *Ura*⁺ phenotypes was followed.

Irradiation: Cells from growing cultures were diluted to appropriate concentrations, plated on solid YPD and UV-irradiated. They were left 5 days at 24° before observation.

Low-density cell suspensions were γ -ray irradiated in a ⁶⁰Co source at a dose rate of 10 Gy/min. Cells were then plated on complete or minimal medium and left 5 days at 24° before observation. All the results presented have been reproduced at least twice.

Flow cytometric analysis of cells: Cells were made rho⁰ by ethidium bromide treatment (SIMON and FAYE 1984). Washed cells were fixed in 70% ethanol, RNase treated and stained with propidium iodide (MANN *et al.* 1992). Flow cytometric analysis was carried out on a Becton-Dickinson (Mountain View, CA) FAC Vantage SEM.

RESULTS

Site-specific mutagenesis of the cysteine-rich domain of *POL3*: The cysteine-rich domain of *POL3* may be important for function because a thermosensitive mutant *pol3-11*, carried a cysteine-to-serine substitution at

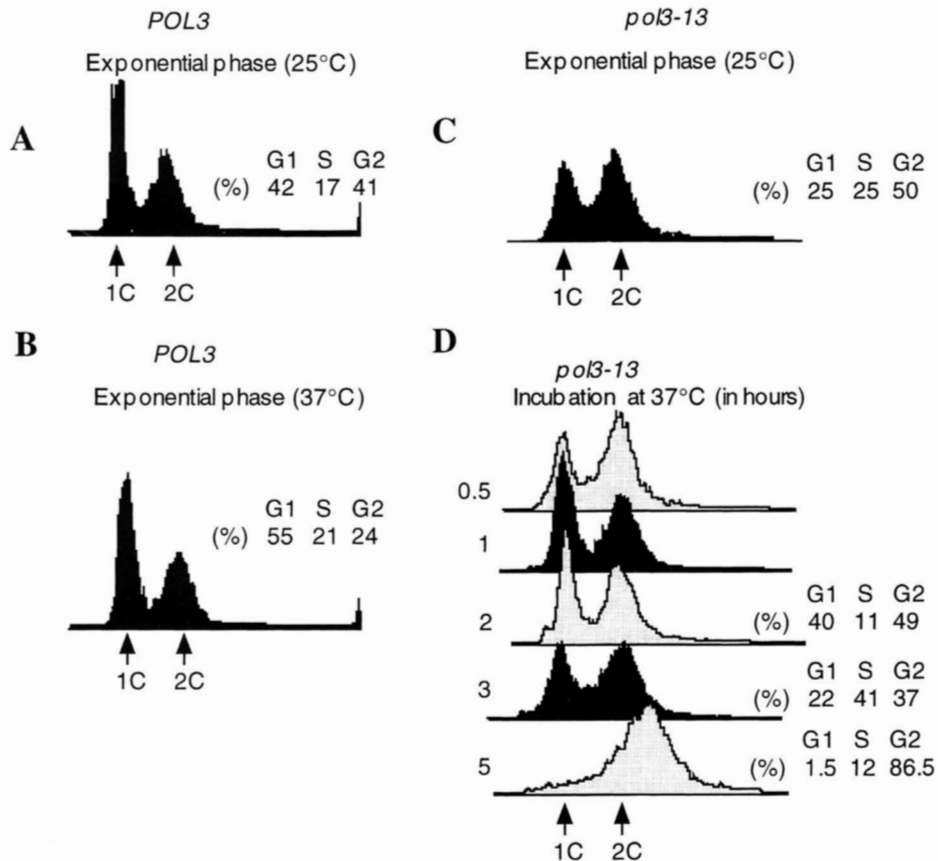


FIGURE 2.—Flow cytometry analysis of cells. Flow cytometry of *POL3* cells in growing conditions in complete medium either at 25° (A) or 37° (B). Flow cytometry of *pol3-13* cells in growing conditions at 25° (C) or grown exponentially at 25° and then incubated at 37° for various periods of time (D). The proportion of G1, S and G2 cells in the populations was calculated with the ModFit LT program according to HEDLEY *et al.* (1993).

residue 1069, previously isolated (GIOT *et al.* 1995) (C1069S) (GIOT *et al.* 1995). Some cysteine-rich domains are known to be able to form zinc finger structures in which cysteine residues coordinate an atom of zinc and are essential to establish the structure of the protein (VALLEE *et al.* 1991). To get some clues about the role of this domain in *POL3*, five of the eight cysteine residues belonging to two putative zinc domains (Figure 1A) were changed by means of oligonucleotide-mediated mutagenesis (KUNKEL *et al.* 1987). The resulting mutant alleles were integrated into strain GF289(501) for analysis. Since *POL3* is essential, this strain contained the wild-type *POL3* gene on a *URA3* plasmid (SIMON *et al.* 1993). Pol3p function was then inferred by testing transformants for their ability to grow in the absence of the wild-type *POL3*.

Analysis of the mutant phenotypes indicated that the cysteine residues are not equivalent. Mutations C1024G and C1059S led to a wild-type phenotype at 25 or 37°, mutations C1027W and C1069N were lethal and the mutant-bearing mutation C1074S (*pol3-13*) was thermosensitive at 30° (Figure 1B). The presence of several essential cysteine residues identifies a new essential domain for DNA polymerase δ . However, it is not known whether these cysteine residues form a classical zinc finger.

Diploid effect for thermosensitivity: To explore further the function of the cysteine-rich domain of *POL3*,

we characterized the *pol3-13* mutant. At permissive temperature (25°), the cell cycle is affected as judged by flow cytometric analysis, although the generation time is the same as that of the *POL3* strain. The proportion of G2 and S phase cells is increased by a factor of 1.2 and 1.5, respectively, as compared to that observed for the *POL3* strain (Figure 2). After a 5-hour incubation at the restrictive temperature (37°), 90% of the *pol3-13* cells exhibit a dumbbell structure, with the nuclear material blocked at the bud neck and an apparent 2C DNA content (Figure 2), which is consistent with the morphology of mutants deficient in DNA replication. The thermosensitivity could be due to the inactivation of Pol3-13p or to the introduction of lethal disorders during replication. To analyze the ability of the mutant to support growth after exposure at the restrictive temperature, we measured the viability of *pol3-13* cells at permissive temperature (24°) after exposing them to 37° for various periods of time. After a lag period, 60% of the haploid cells died per hour of exposure at 37° (Figure 3A). Surprisingly, the *pol3-13/pol3-13* diploid was 1.6 times more sensitive than the isogenic haploid, as defined by the ratio of the inactivation slopes. Other thermosensitive mutants affecting the catalytic domain of *POL3* did not show such a diploid effect (GIOT *et al.* 1995). A similar analysis was difficult to perform with mutant *pol3-11* (C1069S), which modifies the cysteine

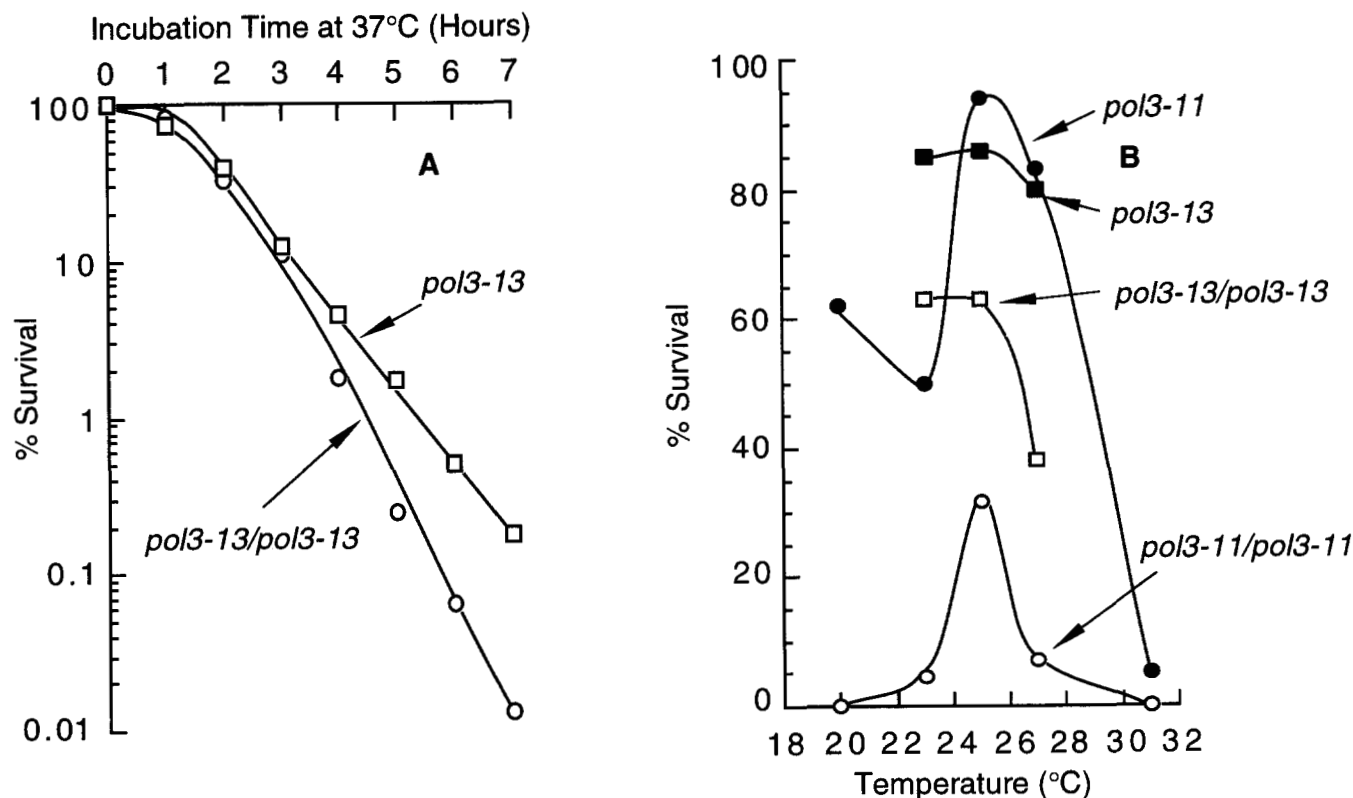


FIGURE 3.—Thermosensitivity of the *pol3-13* and *pol3-11* mutants. (A) *pol3-13* haploid and diploid. Exponential-phase cells were incubated for various periods of time at 37° on YPD plates before their transfer to 25°. □, haploid; ○, diploid. In such a test, wild-type cells are fully resistant. These data are the average of two experiments. (B) *pol3-13* and *pol3-11* haploid and diploid. For each temperature tested, ~40 haploid cells (● and ■) and zygotes (○ and □) were isolated on YPD plates by micromanipulation and their progeny were followed. ■ and □, *pol3-13*; ● and ○, *pol3-11*.

residue nearest to the site of mutation *pol3-13*, because the diploid cells are poorly viable. Instead, we carried out the following experiment: we isolated haploid *pol3-11* cells and *pol3-11/pol3-11* zygotes by micromanipulation and followed their growth into colonies on complete medium at 20, 23, 25, 27 and 31° (Figure 3B). Whereas 92% of haploid cells formed a colony at 25°, only 32% of zygotes did so. The remaining haploid cells or zygotes arrested proliferation after a few divisions. At 23 and 27°, only 5% and 7.5% of zygotes developed a colony, respectively; so the optimum temperature allowing growth of diploid *pol3-11/pol3-11* is very tight compared to haploid *pol3-11*. Altogether these observations indicate that alterations of the cysteine-rich domain induce irreversible damage and reveal a connection between these events and either the ploidy of the cells or the fact that diploids are heterozygous for mating type. To discriminate between these two hypotheses, the formation of diploids *MATa/MATa* and *MAT α /MAT α* was induced by γ -irradiating *pol3-13/pol3-13* cells, and the thermoinactivation of the *MAT* homozygous diploids was determined. The *MATa/MATa* and *MAT α /MAT α* diploids displayed a thermosensitivity similar to that of *MATa/MAT α* cells (data not shown). This result thereby excludes an influence of the mating

type heterozygosity on the diploid effect, and we conclude that *pol3-13* (and *pol3-11*) are sensitive to the presence of homologous chromosomes.

***pol3-13* is sensitive to γ rays:** We have shown previously that a thermosensitive diploid strain heteroallelic for *POL3* (*cdc2-1/cdc2-2*) is deficient in γ -radiation-induced recombination at the restrictive temperature (FABRE *et al.* 1991). To determine whether *pol3-13* strains are deficient in DNA strand break repair at the permissive temperature, the mutant was exposed to methyl methanesulfonate (MMS). Both haploid *pol3-13* and diploid *pol3-13/pol3-13* are unable to grow at 25° in a complete medium containing 0.012% MMS, a concentration that does not inhibit the growth of wild-type cells, suggesting a defect in DNA strand break repair (data not shown).

Both haploid and diploid strains were then subjected to γ rays (Figure 4). Usually, a wild-type haploid is sensitive when irradiated in the G1 phase of the cell cycle but more resistant in the G2 phase (BRUNBORG and WILLIAMSON 1978). A wild-type diploid, containing homologous chromosomes, is relatively resistant in the G1 and G2 phases. Both *pol3-13* haploid and *pol3-13/pol3-13* diploid are sensitive to ionizing radiation. Compared to wild type, the *pol3-13* haploid is only affected in the

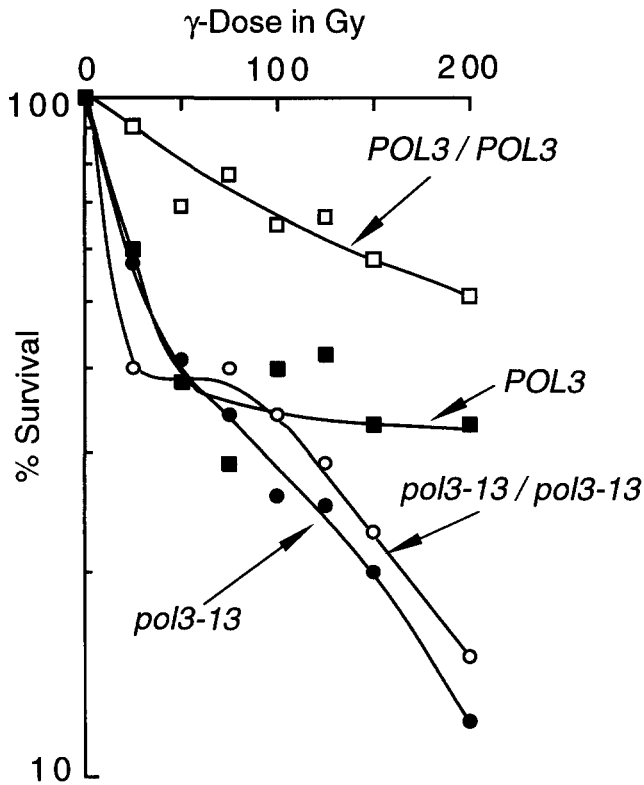


FIGURE 4.—Response of *pol3-13* to γ irradiation. Exponential-phase cultures were irradiated with γ rays and incubated on YPD plates for 5 days at 24° before colonies were counted. Wild-type strains haploid GF260 (■) and diploid DG146 (□). *pol3-13* haploid (●) and diploid (○).

G2 phase. The *pol3-13/pol3-13* diploid is highly sensitive in the G1 phase. Taken together, these results indicate that the *pol3-13/pol3-13* cells have a strong repair defect after γ irradiation in G1 phase and that both haploid and diploid cells have a partial repair defect of γ -induced lesions when in G2 phase at permissive temperature.

***pol3-13* is defective for induced recombination:** Sensitivity to induced DNA strand breaks may reflect alteration(s) in mutagenic or recombinogenic DNA repair, but this sensitivity may be also due to indirect effects. For instance, alleles of *POL1* and *POL3* (named *hpr3* and *hpr6*, respectively) cause sensitivity to MMS and a large increase in spontaneous recombination (AGUILERA and KLEIN 1988). Association of both phenotypes was attributed to an accumulation of lesions in the DNA that are recombinational substrates and leave the cells unable to recover from an increased number of lesions due to the damaging agent. To investigate whether *POL3* was directly involved in induced recombination, we compared the ability of *pol3-13/pol3-13* and wild-type diploid cells to recombine at 24°. To do so, we followed spontaneous rates and induced frequencies of Arg⁺ recombinants formed in a heteroallelic *arg4B/arg4R* diploid (NICOLAS *et al.* 1989). In the *pol3-13/pol3-13* strain,

the spontaneous recombination rate was comparable to that of the wild-type control strain (7×10^{-6} and 5.5×10^{-6} Arg⁺ per generation, respectively), suggesting that the *pol3-13/pol3-13* mutant is not affected in spontaneous recombination. However, among the survivors, γ irradiation did not induce intragenic recombination in the *pol3-13/pol3-13* diploid, although the wild-type diploid responded linearly to the applied doses (Figure 5A). To show that this defect was not restricted to the repair of lesions induced by ionizing radiation, both strains were exposed to UV irradiation and the frequency of recombinants was established. The same defect was observed since increasing UV doses did not induce intragenic Arg⁺ recombinants in a *pol3-13/pol3-13* diploid, whereas the number of Arg⁺ recombinants increased in direct ratio to UV doses in a wild-type diploid (Figure 5B). These data indicate that *pol3-13* mutants are defective for radiation-induced recombinational repair and that spontaneous and induced recombination follow different pathways.

***pol3-13* is defective for induced mutagenesis:** The implication of *POL3* in recombinational repair suggests that *POL3* may be involved in other DNA repair pathways. To investigate this possibility, we submitted *pol3-13* haploid and diploid strains and isogenic wild-type strains to increasing UV doses at 24°. Mutant *pol3-13* is clearly more sensitive than the wild-type strain (Figure 6A), indicating a defect in repair of UV-induced lesions. We also observed that the diploid effect is altered since diploid *pol3-13/pol3-13* was as sensitive as haploid *pol3-13*, although a wild-type diploid is more resistant than a wild-type haploid. This result is consistent with the idea that the *pol3-13/pol3-13* diploid is unable to repair lesions through recombination between homologous chromosomes. Upon testing, we observed that the *pol3-11* mutant is UV sensitive as well, whereas the *pol3-14* mutant, which is altered in the Pol3p catalytic domain (GIOT *et al.* 1995), is not sensitive to UV irradiation at the permissive temperature (data not shown). This indicates that UV sensitivity may be specific to mutations that affect the cysteine-rich domain of Pol3p.

UV irradiation introduces lesions that are repaired by both nucleotide excision repair and error-prone repair processes (FRIEDBERG *et al.* 1995). The efficiency of the error-prone repair pathway is easily followed by looking for the appearance of canavanine-resistant colonies before and after UV irradiation. The spontaneous mutation to canavanine resistance (Can^R) displayed a five-fold increase in *pol3-13* when compared to a haploid wild-type strain (data not shown). This indicates that *pol3-13* is a weak mutator compared to the effect of mutations affecting the exonuclease domain (SIMON *et al.* 1991) or the catalytic domain (GIOT *et al.* 1995), which resulted in 500- and 20-fold increases in spontaneous mutagenesis, respectively. Submitted to increasing UV doses, a wild-type strain responds by an increase in the frequency of canavanine-resistant mutants per

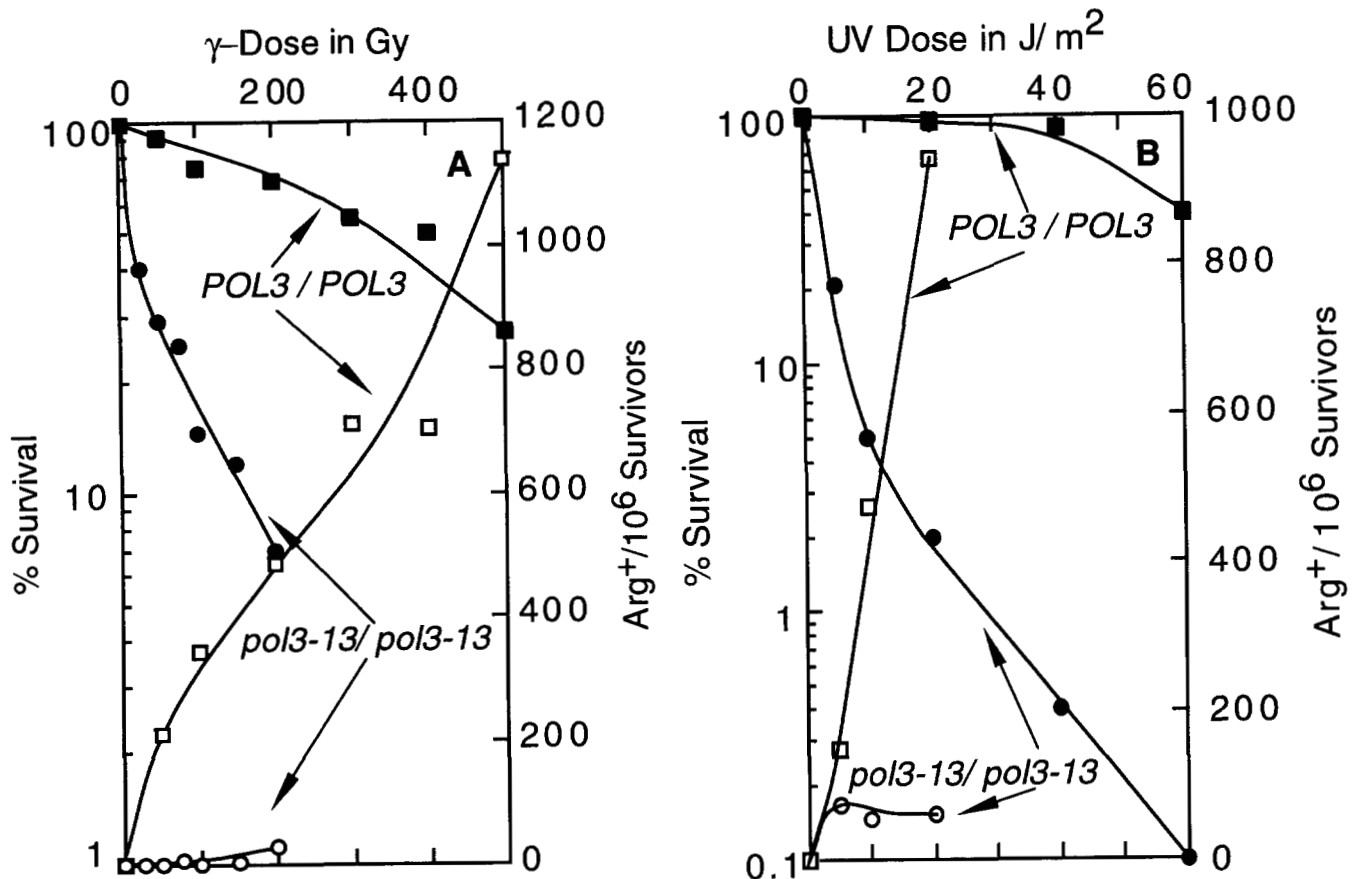


FIGURE 5.—Recombination induced by irradiation. Exponential-phase cultures were irradiated with (A) γ rays or (B) UV rays, plated on YPD to estimate survival or synthetic complete media lacking arginine and left 5 days at 24°. Right ordinate (\circ and \square): numbers of Arg⁺ colonies per 10⁶ survivors. Left ordinate (\bullet and \blacksquare): survival (in percent). \square and \blacksquare , *POL3/POL3 arg4B/arg4R*; \circ and \bullet , *pol3-13/pol3-13 arg4B/arg4R*. \bullet and \blacksquare , survivals; \circ and \square , induced Arg⁺ convertants (the background frequencies have been subtracted; they were between $36\text{--}56 \times 10^{-6}$ and $63\text{--}190 \times 10^{-6}$ for *POL3 POL3/POL3* and *pol3-13/pol3-13* strains, respectively, according to experiments).

survivor. Our results show that this induction is severely diminished in the *pol3-13* strain (Figure 6B). This suggests that induced error-prone repair is defective in *pol3-13*.

Epistasis analysis is often a useful means toward identifying genes that act in the same functional pathway (COX and GAME 1974). The above mentioned results suggest that *POL3* is required for error-prone repair. We have investigated genetic interactions between *pol3-13* and mutations in *RAD18* and *REV3*, which act in the error-prone repair pathway (FRIEDBERG *et al.* 1991). Interestingly, the double mutant *pol3-13 rad18* Δ is able to better tolerate restrictive temperature than *pol3-13* (Figure 7), suggesting that Rad18p is detrimental for DNA replication in the *pol3-13* mutant. The *rad18* Δ alone was more UV sensitive than the *pol3-13* mutant, whereas the double mutant *pol3-13 rad18* Δ was as sensitive as *rad18* Δ (Figure 8A). Thus, *rad18* Δ is epistatic to *pol3-13*. Rev3p is a nonessential DNA polymerase that is supposed to bypass lesions in error-prone repair (MORRISON *et al.* 1989; NELSON *et al.* 1996). Like *rad18* Δ , *rev3* Δ is also epistatic to *pol3-13*, at least for

cells in the G1 phase, the most sensitive phase of *rev3* Δ mutants, suggesting that both DNA polymerases participate in error-prone repair (Figure 8A).

Since *POL3* appears to act in error-prone repair, we might expect an additive or synergistic effect by associating *pol3-13* with a mutation defective in the excision repair pathway such as *rad1* Δ . To test this hypothesis we compared the UV sensitivity of a double mutant *pol3-13 rad1* Δ with each single mutant (Figure 8B). The *pol3-13 rad1* Δ double mutant was more sensitive to UV low doses than *rad1* Δ or *pol3-13* separately (synergistic effect). This result indicates that *pol3-13* and *rad1* Δ are defective in different pathways, which is consistent with the role of *POL3* in error-prone and recombinational repair.

Recombination is essential to *pol3-13*: Different combinations of mutations defective in recombinational repair show a dramatic effect on the survival of *pol3-13*. Double mutants *pol3-13 rad50* Δ , *pol3-13 rad52* Δ and *pol3-13 rad54* Δ are not viable (data not shown). Haploids *pol3-13 rad51* Δ were obtained at a low frequency and grew slowly on complete medium. This double mu-

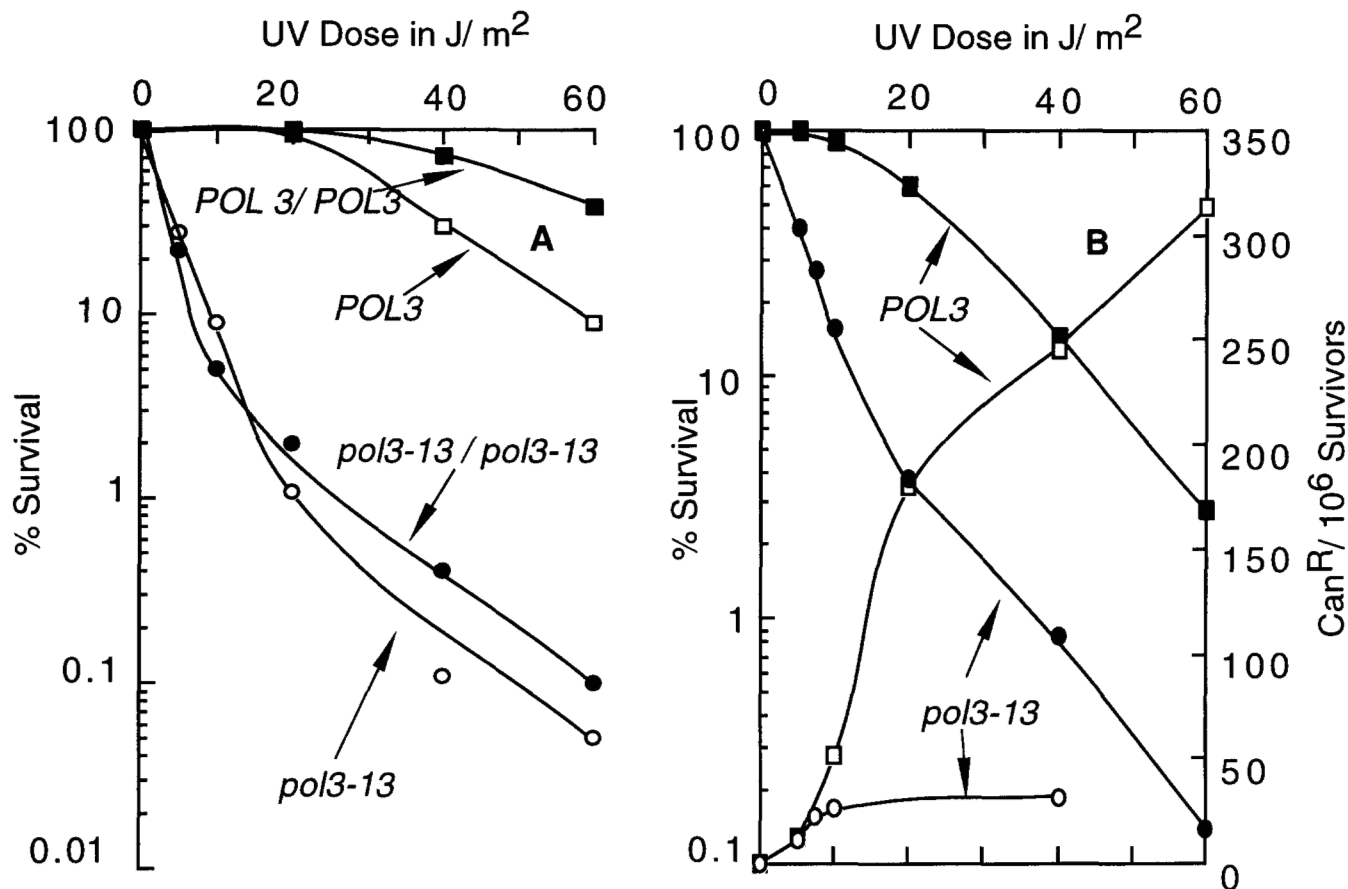


FIGURE 6.—Responses of *pol3-13* to UV irradiation. Exponential-phase cultures were irradiated with UV rays. (A) Colonies were counted after 5 days on YPD at 24°. Wild-type strains haploid GF260 (□) and diploid DG146 (■). *pol3-13* haploid (○) and diploid (●). (B) Survival and forward induction Can^R mutations were estimated by plating UV-irradiated cells on YPD and synthetic complete plates containing canavanine and lacking arginine, respectively. Right ordinate: frequency of canavanine-resistant mutants per 10⁶ survivors. Left ordinate: survival (in percent). □ and ■, wild-type haploid strain; ○ and ●, *pol3-13* strain. ● and ■, survival; ○ and □, induced Can^R mutants (the background frequencies have been subtracted).

tant has a synergistic response to UV irradiation compared to each single mutant (Figure 8C), which is consistent with the participation of *POL3* in the error-prone DNA repair pathway. The synthetic lethality with mutants involved in recombination indicates that in *pol3-13* DNA lesions have to be processed in the recombinogenic pathway or that the proteins involved in the recombinational process belong to a complex needed for DNA replication. Furthermore, *rad18*Δ suppresses the lethality of *pol3-13 rad50*Δ and *pol3-13 rad52*Δ double mutants, although they are still thermosensitive (data not shown). In addition the *pol3-13 rad51*Δ *rad18*Δ triple mutant grows as well as the single mutant *pol3-13* (data not shown). Thus, the *rad18*Δ mutation suppresses the recombination dependency of *pol3-13*, perhaps because Rad18p channels DNA lesions into the recombinational repair pathway that is essential in the *pol3-13* context.

Extragenic suppressors of *pol3-13* identify the Polδ-associated protein p55: The identification of *rad18*Δ as a partial suppressor of the thermosensitivity of *pol3-13* encouraged us to search for extragenic suppressors

of mutation *pol3-13*. Spontaneous *pol3-13* revertants able to grow at 37 or 32° were isolated. Among them, 11 extragenic suppressors were obtained, two of which (*sdp5-15* and *sdp5-20*) were thermoresistant up to 37°. By complementation tests, all suppressors appeared to belong to the same group and were allelic to *sdp5-1*, an extragenic suppressor of *pol3-11* (Giot et al. 1995). The fact that *sdp5-1* and *sdp5-15* were able to suppress the thermosensitivity of both *pol3-11* and *pol3-13*, but not that of *pol3-14*, suggests that this suppressor is specific for mutations located in the cysteine-rich domain of *POL3*. When exposed to UV irradiation, *pol3-13 sdp5-15* was as resistant as a wild-type strain (data not shown). Thus, *sdp5-15* also suppresses the *pol3-13* defect in DNA repair. In addition, the triple mutant *pol3-13 sdp5-15 rad51*Δ, which grows as well as *pol3-13* at permissive temperature, was UV sensitive and thermosensitive like *pol3-13* (data not shown). The suppressing ability of *sdp5-15* is then recombination dependent. Genetic analysis showed that *SDP5* is not allelic to *RAD18* (data not shown).

The *sdp5-15* gene was cloned by virtue of its domi-

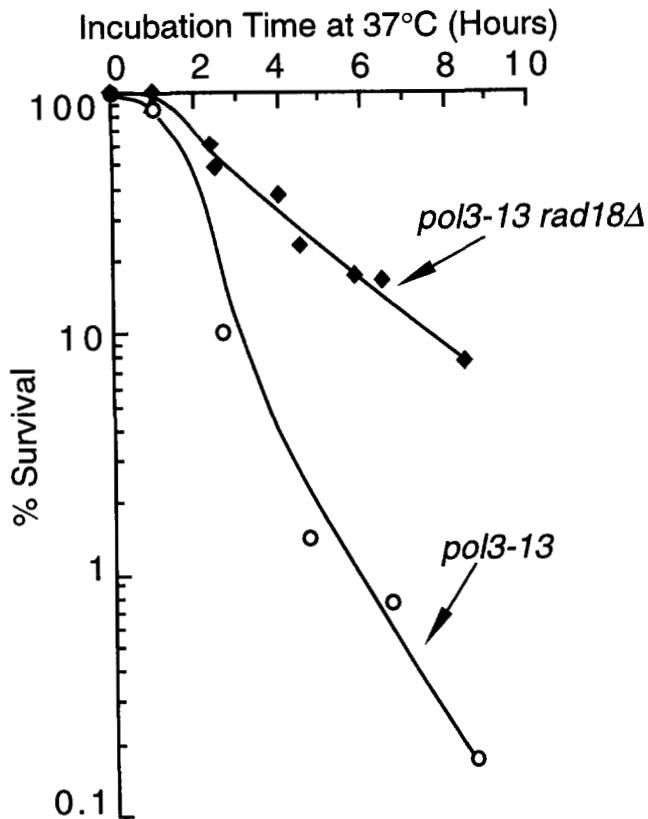


FIGURE 7.—*rad18Δ* partially suppresses the thermosensitivity of *pol3-13*. Exponential phase cells were incubated for various periods of time at 37° on YPD plates before their transfer to 25°. ○, *pol3-13*. ●, double mutant *rad18Δ pol3-13*.

nance: homozygous *pol3-13/pol3-13 sdp5/sdp5* diploids are as thermoresistant up to 35° as the heterozygous diploid strain *pol3-13/pol3-13 sdp5-15/SDP5*. Eleven plasmids from a genomic library constructed from mutant *pol3-13 sdp5-15* were able to suppress the thermosensitivity of *pol3-13* at 35° (data not shown). All plasmids contained overlapping inserts, harboring a common 2.1-kb *EcoRV* DNA fragment (data not shown); the latter was sequenced. The fragment contains a 487 amino acid open reading frame (M_r : 55,261 D) identical to *HYS2* (Swiss-Prot accession no. P46957). *HYS2* has been recently identified through a screen for mutants hypersensitive to hydroxyurea (SUGIMOTO *et al.* 1995). It is an essential gene, and conditional mutants display the phenotypes expected for a defect in DNA replication. Furthermore, one allele presents a defect in response to UV irradiation (SUGIMOTO *et al.* 1995).

To confirm the identity between *SDP5* and the cloned gene, we inserted *URA3* near the *SDP5* locus (see MATERIALS AND METHODS) and crossed the resulting strain with both *pol3-13 sdp5-15* and *pol3-11 sdp5-1* double mutants. In both cases, thermosensitivity segregated with the *Ura*⁺ phenotype (30 and 16 tetrads studied, respectively), indicating that the cloned gene is (or is near) the one affected by mutations *sdp5-1* or *sdp5-15*.

Comparison of the amino acids sequence with the Swiss Prot release 35 showed (Figure 9) a low but significant homology (30% of identity over the full-length proteins) with two polypeptides encoding the small subunit (p50) of bovine and human DNA polymerase δ (ZHANG *et al.* 1995a). Interestingly, the sequence of the mutant allele *sdp5-15* predicts only one change in a conserved domain at the position 358, which is a lysine in *SDP5* and a glutamic acid in *sdp5-15* (data not shown). We suggest that *SDP5* codes for the p55 protein (BAUER and BURGERS 1988) associated with Pol3p in *S. cerevisiae*.

DISCUSSION

Pol δ is required for the repair of induced DNA damages: Mutant *pol3-13* seems to be altered in DNA replication since the proportion of its S and G2 phase cells is increased compared to that of *POL3* cells. However, the generation times of *pol3-13* and *POL3* are equal (data not shown) so we should not expect that the *pol3-13* generated replication defects to be so drastic. The synthetic lethality of *pol3-13* with *rad50Δ* or *rad52Δ* suggests that these defects require the recombinogenic repair pathway to be processed. The nearly normal level of spontaneous DNA mutagenesis and recombinogenesis in mutant *pol3-13* also means that these processes are active but not exacerbated by the *pol3-13* replication defects.

The question is then raised as to whether these spontaneous defects may interfere with the DNA repair of induced lesions. The answer is not easy for there is no clear relationship between spontaneous lesions left by various mutated genes and the sensitivity to radiation. For instance, *mut7-1*, an allele of *POL3* (VON BORSTEL *et al.* 1993), is a weak mutator and is not UV sensitive; *pol3-4DA*, which is mutated in the editing exonuclease domain of *POL3*, though exhibiting a strong mutator phenotype (SIMON *et al.* 1991), is very weakly UV sensitive (M. SIMON, unpublished result); the *rad3-102 (rem1-1)* mutant (GOLIN and ESPOSITO 1977) is also a strong mutator and recombinator but is not radiation sensitive. To explain the sensitivity of *pol3-13* to DNA-damaging agents, one may suppose that the *pol3-13* cells have only a low proficiency to repair DNA lesions that are in the vicinity of *pol3-13*p-generated replication defects. This deficiency could be accounted for by one of two models: either the replication complex containing the *pol3-13*p is blocked by DNA lesions, thereby impeding the repair pathways in an indirect way, or *pol δ* is a component of repair complexes, in particular those dealing with mutagenesis or recombinogenesis. We consider this second model as the more plausible one with regard to our results and to data obtained with higher eukaryotes: (1) calf thymus PCNA dramatically enhances the ability of calf thymus *pol δ* to bypass *in vitro* UV-induced lesions (O'DAY *et al.* 1992) and (2) *pol δ* appears to be involved

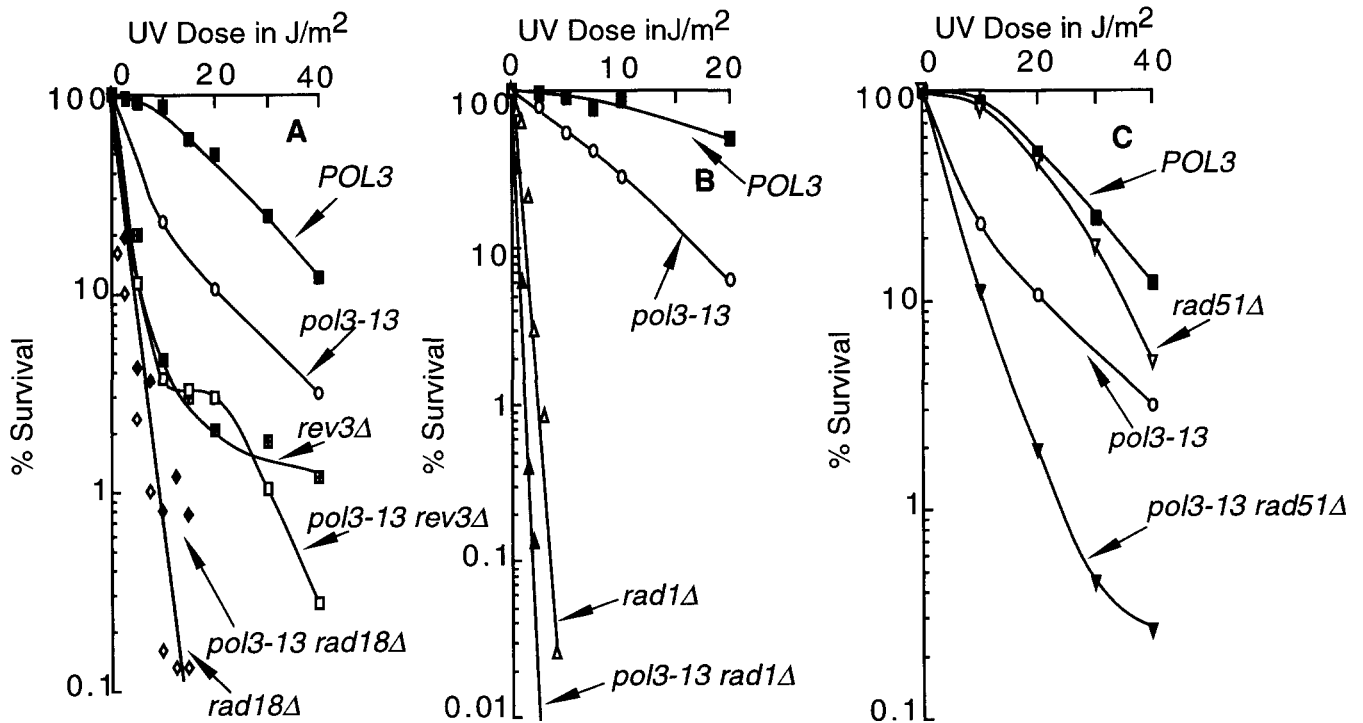


FIGURE 8.—Epistasis analysis with *rad18Δ*, *rev3Δ*, *rad1Δ* and *rad51Δ*. Exponential-phase cultures were UV irradiated and then left 5 days on YPD plates at 24° before counting survivors of (A) haploid *rad18Δ* (open diamonds), *rad18Δ pol3-13* (closed diamonds), *rev3Δ* (crossed squares) and *pol3-13 rev3Δ* (squares); (B) haploid *rad1Δ* (Δ), and *pol3-13 rad1Δ* (▲); (C) haploid *rad51Δ* (∇) and *pol3-13 rad51Δ* (▼). Wild-type and *pol3-13* haploids are represented by ■ and ○, respectively, in the graphs.

in DNA repair of UV-induced lesions in mammalian cells (DRESLER and FRATTINI 1986; ZENG *et al.* 1994).

Both Pol δ and Pol ϵ polymerase activities are enhanced by PCNA *in vitro* (BURGERS 1991). Since PCNA is also required for DNA repair synthesis (SHIVJI *et al.* 1992), both polymerases are candidates for DNA repair synthesis. Whatever the nature of the defect in the DNA metabolism at 24°, our results suggest that Pol δ is involved in the repair of radiation-induced DNA lesions *in vivo*.

The differential effect of the *pol3-13* mutation in repair processes suggest that spontaneous and induced DNA lesions have different requirements for replication components participating in their resolution. We postulate that DNA polymerase δ is recruited for DNA repair of radiation-induced lesions. One possibility is that the *pol3-13* mutant is able to repair spontaneous but not radiation-induced lesions, supposing that Pol δ is the only DNA polymerase for DNA repair. A second possibility is that another DNA polymerase is involved specifically in the repair of spontaneous lesions while Pol δ is recruited after irradiation. This could account for the conflicting reports on the assignment of Pol δ and Pol ϵ in the different DNA repair pathways. In fact, Pol ϵ has been isolated as a component of the recombinational apparatus in cell extracts (JESSBERGER *et al.* 1993), while we show that Pol δ is needed for induced recombination. Pol ϵ is also needed for base excision repair *in vitro*

(WANG *et al.* 1993), while other studies have shown Pol δ being needed for cellular response to MMS (BLANK *et al.* 1994) and to UV damage *in vitro* (ZENG *et al.* 1994) and *in vivo* (this study). In agreement with this model are the recent findings that Pol δ directly binds to PCNA (ZHANG *et al.* 1995b), which is targeted to the nucleus, to interact with chromatids damaged by UV or γ irradiation (MIURA *et al.* 1992, 1996). Possibly, PCNA recruits Pol δ to repair irradiation-induced DNA lesions.

Pol δ acts in the error-prone repair pathway: The error-prone repair pathway bypasses lesions by introducing mutations on the newly synthesized strand (FRIEDBERG *et al.* 1991). The Rev3p DNA polymerase is associated with mutagenesis induced by a variety of DNA-damaging agents (FRIEDBERG *et al.* 1995), and it was argued that the Rev3p DNA polymerase functions only in translesion synthesis (MORRISON *et al.* 1989). Our results suggest that *pol3-13* as well as *pol3-13* (Rev3p) is also involved in this process.

Genetic interaction between recombination and error-prone repair: Our observations implicate *POL3* in induced recombination (FABRE *et al.* 1991; this study). Deletions of genes involved in recombination (*RAD50*, *RAD52* and *RAD54*) show synthetic lethality with *pol3-13* (data not shown). Although the recombination processes are essential in *pol3-13* haploids, they are not sufficient to perform recombinational DNA repair in diploids. We suggest that the recombination complexes

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SDP5p      MDALLTKFNEDRSLQDENLSQPRTR----VRIVDNLYNKSNNPFCQCYK
p50HUMAN  M-----FSEQAAQRAHTLLSPPSANNATFARVVPVATYTNSSQPFRL--G
          * * * * *
SDP5p      KRDRYGSQYVYHIYQYRLKTRFRERVLKCECDKRWDAFTLNGQLVLLKDKVLD
p50HUMAN  ERSFSRQYAHYIATRLIQMRPFLENRAQQHWGSGVGVK-----KLCE
          * * * * *
SDP5p      IQGNQPCWCVGSIYCEMKYKPNVLDEVINDTYGAPDLTKS-YTDKKEGGSD
p50HUMAN  LQPEEKCCVVGTLFKAMPLQPSILREVSEHNLLPQPPRSKYIHPD---D
          * * * * *
SDP5p      EIMLEDESGRVLVVGDFIRSTPFFITGVVVGILGMEAEAGTFQVLDICYP
p50HUMAN  ELVLEDELQRILKLGKTI-DVSKLVTGTVLAVFGSVRDDGKFLVEDYCFAD
          * * * * *
SDP5p      PLPQNPFPAIATPCPTRGKIALVSGMLNNTSPDRLLRLEILREFLMGRI
p50HUMAN  LAPQKP-APPLDTRDF---VLLVSGGLGGGGESLLGTQLLVVVVTGQL
          * * * * *
SDP5p      NNKIDDISL--IGRLLICGNSVDFDIKS--VNKDELMLSLTEFS-----
p50HUMAN  GDEGEQCSAAHVSRLVILAGNLLSHSTQSRDSINKAKYLTKKTAASVEAV
          * * * * *
SDP5p      KFLHNIL----PSISVDIMPGTNDPSDKSLPQQPFHKSFLDKSLESYFNG
p50HUMAN  KMLDEILLQLSASVPVDVMPGEFDPNTYTLPPQPLHPCMF--PLATAYS-
          * * * * *
          K
SDP5p      SNKEILNLVNTNPFYEFYVNGVDVLA VSGENINDICKYVIPSNDNGESENKV
p50HUMAN  ---TLQLVNTNPFYQATIDGVRFLGTISGQNVSDIFRY-----
          * * * * *
SDP5p      EEAGESNDFKDDIEHRLDLMCEMTKWNLIAPTADTLWCYFPYTDKDFPVL
p50HUMAN  -----SSMEDHLEILELWLRVHRHISPTADTLGCYFPYKTDPFIFP
          * * * * *
SDP5p      KWPVYIVANQPYFGTRVV-EIGGKNIKIISVPEFSSTGMIILLDLETLE
p50HUMAN  ECPHYVFCGNTPSFGSKIIRGPEQTVLLVTVPDPSATQACLVNLRSLA
          * * * * *
SDP5p      AETVK-----IDI 487
p50HUMAN  CQPIFSFGFAEDDGLGLGP 469

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FIGURE 9.—Comparison of Sdp5p and human p50 protein sequences. The sequences of human p50 (ZHANG *et al.* 1995a) and of Sdp5p/Hys2p (SUGIMOTO *et al.* 1995) are compared with the sequence of *sdp5-15*. *Identical amino acid residues. The mutation found in *sdp5-15* is noted at position 358.

involved in postreplicative events are different from those performing recombinational repair where the integrity of Pol δ is necessary. The recombination that is essential to the *pol3-13* mutant is linked to the Rad18p function, since deletion of the corresponding gene bypasses the recombination requirement. In addition, *rad18 Δ* (as well as *sdp5-15*) partially suppresses the thermosensitivity of *pol3-13* in a recombination dependent manner, *i.e.*, *pol3-13 rad18 Δ rad51 Δ* is more thermosensitive than *pol3-13 rad18 Δ* (Figure 7 and data not shown). Taken together, these observations indicate that Rad18p acts at an early step and that a connection does exist between Rad18p and the recombinational apparatus. Similar genetic interactions have been shown between error-prone repair and the Rad51p-dependent recombination repair (ABOUSSEKHRA *et al.* 1992). Since Rad18p interacts with single-stranded DNA (BAILLY *et al.* 1994), we propose that, in mutant *pol3-13*, the binding of Rad18p on DNA lesions, or structures, channels the error-prone substrates toward recombination. Because *pol3-13* is partially defective in this pathway, Rad18p is detrimental. In contrast, the absence of Rad18p could enable the cell to treat the lesions by a pathway unaffected in *pol3-13*.

Interaction between Pol δ and Sdp5p: Defects in both error-prone and recombinational repair were detected only in mutants affecting the cysteine cluster of *POL3*. None of the thermosensitive mutants located in the catalytic domain of Pol δ (GIOT *et al.* 1995) displayed UV

or MMS sensitivity or any diploid effect for temperature. Thus, the C-terminal domain of *POL3* seems to be sensitive with regard to the function of *POL3* in DNA repair. However, *pol3-13* and *pol3-11* are also thermosensitive for growth, reflecting the essential role of this domain for the function of *POL3* in DNA replication.

Extragenic suppressors of *pol3-13* and *pol3-11* identified *SDP5/HYS2*, which presents significant homology with the p50 subunit of bovine and human DNA polymerases δ . The suppression is specific for mutations affecting the cysteine-rich domain. We propose that p55 binds the cysteine-rich domain of *POL3* in accord with the co-purification of p55 with Pol3p (BURGERS and BAUER 1988). Two alleles, *sdp5-1* and *sdp5-15*, also suppress the defect in DNA repair and an allele of *HYS2* displays hypersensitivity to UV irradiation (SUGIMOTO *et al.* 1995). Thus, Sdp5p could be associated with Pol3p during DNA repair synthesis as PCNA seems to be.

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