Curr Genet (2000) 38: 178–187

ORIGINAL PAPER

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POL32, a subunit of the *Saccharomyces cerevisiae* DNA polymerase δ, defines a link between DNA replication and the mutagenic bypass repair pathway

Received: 25 May 2000 / Accepted: 3 July 2000

Abstract Pol32 is a subunit of *Saccharomyces cerevisiae* DNA polymerase δ required in DNA replication and repair. To gain insight into the function of Pol32 and to determine in which repair pathway *POL32* may be involved, we extended the analysis of the *pol32*Δ mutant with respect to UV and methylation sensitivity, UV-induced mutagenesis; and we performed an epistasis analysis of UV sensitivity by combining the *pol32*Δ with mutations in several genes for postreplication repair (*RAD6* group), nucleotide excision repair (*RAD3* group) and recombinational repair (*RAD52* group). These studies showed that *pol32*Δ is deficient in UV-induced mutagenesis and place *POL32* in the error-prone *RAD6*/*REV3* pathway. We also found that the increase in the *CAN1* spontaneous forward mutation of different *rad* mutators relies entirely or partially on a functional *POL32* gene. Moreover, in a two-hybrid screen, we observed that Pol32 interacts with Srs2, a DNA helicase required for DNA replication and mutagenesis. Simultaneous deletion of *POL32* and *SRS2* dramatically decreases cellular viability at 15 °C and greatly increases cellular sensitivity to hydroxyurea at the permissive temperature. Based on these findings, we propose that *POL32* defines a link between the DNA polymerase and helicase activities, and plays a role in the mutagenic bypass repair pathway.

Key words *POL32* · *SRS2* · DNA repair · *Saccharomyces cerevisiae*

Communicated by L. A. Grivell

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Introduction

DNA polymerase (Pol) δ is the major replicative DNA polymerase in eukaryotic cells, playing a role in bulk DNA replication (Burgers 1998). It is also the primary DNA polymerase for most DNA repair pathways (Budd and Campbell 1997; Burgers 1998). *Saccharomyces cerevisiae* Pol δ has recently been defined and comprises three subunits with apparent sizes of 125, 58 and 55 kDa, encoded by the *POL3*, *POL31* (*HYS2*, *SDP5*) and *POL32* genes, respectively (Gerik et al. 1998). Both Pol3 and Pol31 are essential for cell growth (Boulet et al. 1989; Sugimoto et al. 1995). Pol3 is the catalytic subunit; and the function of Pol31, the second subunit, is not well known. Mutations in *POL31* can either cause sensitivity to the replication inhibitor hydroxyurea (HU) or suppress the temperature sensitivity of mutations in the catalytic subunit (Sugimoto et al. 1995; Giot et al. 1997). Pol32 is under investigation in the present study. Previous experiments in our and Burgers' laboratories have shown that *pol32*Δ mutants are viable but cold-sensitive for growth (Huang et al. 1997, 1999; Gerik et al. 1998). The *pol32*Δ mutants display both replication and repair defects, manifested as a higher proportion of large-budded cells with a single but duplicated DNA mass at the mother-bud neck, increased sensitivity to HU and increased sensitivity to ultraviolet (UV) radiation and methylation damage (Gerik et al. 1998; Huang et al. 1999). They are also defective for induced mutagenesis, suggesting the involvement of Pol32 in error-prone repair (Gerik et al. 1998). In addition, Pol32 interacts with several proteins involved in DNA replication and repair, including Pol1, the catalytic subunit of Pol α, Pol3, Pol31 and the proliferating cell nuclear antigen (PCNA; Gerik et al. 1998; Huang et al. 1999). In vitro, DNA synthesis mediated by Pol δ lacking Pol32 is inefficient and is characterized by frequent pausing. It has been proposed that interactions between the Pol3 and/or Pol32 subunits are essential for establishing a productive PCNA-polymerase complex. Physical

interaction(s) between PCNA and Pol32 may stabilize this complex, particularly during the replication of secondary structures in the DNA template (Burgers and Gerik 1998).

In the present work, we explored the repair defect of *pol32* cells. In yeast, genes that influence cellular responses to DNA damages fall into three major groups, initially defined by genetic and epistasis analysis and usually considered to control three different types of DNA repair (Friedberg et al. 1995). Genes in the *RAD3* epistasis group are mainly involved in the repair of DNA damage by nucleotide excision repair (NER). Genes in the *RAD52* epistasis group function in recombinational repair and are mainly involved in the repair of double-strand breaks in DNA. Genes in the *RAD6* epistasis group mediate postreplication repair (PRR), which allows for the replicative bypass of damaged DNA templates via the error-free and error-prone mechanisms (Friedberg et al. 1995).

Many lines of genetic evidence support the role of Rad6 and Rad18 in both the error-free and error-prone mutagenesis pathways (Friedberg et al. 1995). The *RAD6* gene encodes a ubiquitin-conjugating enzyme (Jentsch et al. 1987). To carry out its function, Rad6 forms a tight complex with Rad18, a DNA-binding protein (Bailly et al. 1997). The Rad18 protein may therefore target Rad6 to sites of DNA damage. Some members of the *RAD6* group are involved primarily (or exclusively) either in the error-free or in the error-prone pathway. For example, Rad5, Rad30, Mms2 and PCNA function in the error-free bypass of UV-induced DNA damage. Rad5 is a DNA-dependent adenosine triphosphatase (Johnson et al. 1994). Rad30, homologous to *Escherichia coli* DinB and UmuC proteins (McDonald et al. 1997), is a DNA polymerase (Pol η) that can bypass a thymine–thymine *cis-syn* cyclobutane (T–T) dimer in an error-free manner by inserting two A residues opposite the two Ts of the dimer (Johnson et al. 1999a, b). Genetic data suggest that Rad5 and Rad30 constitute alternatives to the Rad6/Rad18-dependent pathways for the error-free bypass of UV-damaged DNA templates (McDonald et al. 1997). *MMS2* encodes a ubiquitin-conjugating enzyme-like protein (Broomfield et al. 1998). In addition two mutants, *rad6Δ1–9* (which has an N-terminal deletion of Rad6) and *pol30–46* (which is a PCNA mutant) are known to be defective in error-free PRR (Watkins et al. 1993; Torres-Ramos et al. 1996). In contrast, Rev1, Rev3 and Rev7 function in the error-prone bypass of UV-induced DNA damage. Rev1 has a deoxycytidyl transferase activity that transfers a deoxycytidine 5′-monophosphate residue dCMP opposite G, A or an abasic site (Nelson et al. 1996a). Rev3 and Rev7 associate to form Pol ζ, which shows ability to bypass a *cis-syn* T–T dimer (Nelson et al. 1996b). Pol ζ is considered to be responsible for practically all mutagenesis resulting from DNA damage in budding yeast (Lawrence and Hinkle 1996). In addition, genetic evidence indicates some role for Pol δ in error-prone bypass replication, as damage-induced mutagenesis is defective in *pol3–13* mutants and *pol32Δ* mutants (Giot et al. 1997; Gerik et al. 1998).

The mutagenic replisome may be extremely complex. An additional protein, Srs2, may also be involved in the error-prone pathway (Friedberg and Gerlach 1999). The *SRS2* gene, which codes for a helicase with a 3′-to-5′ polarity (Rong and Klein 1993), plays a complex role in the choice of repair pathway for damaged DNA (Aboussekhra et al. 1989; Schiestl et al. 1990). Damage-induced mutagenesis is defective in *srs2* mutants and the high spontaneous mutagenesis in *rad5* and *rad18* mutants is Srs2-dependent (Aboussekhra et al. 1989; Liefshitz et al. 1998). A recent study indicated that Srs2 plays a role both in DNA replication and in RNA polymerase I transcription (Lee et al. 1999).

To gain an insight into the biological role of Pol32, we performed a series of genetic analyses suggesting that Pol32 defines a link between DNA replication and the mutagenic bypass repair pathway (*RAD6*/*REV3*).

Materials and methods

Strains, media and general methods

The *S. cerevisiae* strains used in this study are listed in Table 1. All strains (except YTS7 and YTS31) derive from FY1679 (Thierry et al. 1995). Disruption mutations were introduced by standard lithium acetate transformation-based gene disruption techniques and in some cases by crossing mutant derivatives to isogenic derivatives of FY1679. The disruption fragments were usually transformed into diploid FY1679C or HD004, and the disruptants were selected on appropriate dropout plates. All gene disruptions were confirmed by genomic Southern blotting or by PCR analysis using primer pairs that allowed amplification of both the wild-type and the mutant alleles. Haploid disruptant strains were obtained by sporulation. The *POL32* deletion cassette, containing a kanMX4 selection module, was described previously (Huang et al. 1997). Strains YTS7 and YTS31 were obtained from L. Prakash (Torres-Ramos et al. 1996) and were transformed with the *POL32* deletion cassette to generate gene disruptions in this strain background. Plasmids pDG315 (*rad6Δ::LEU2*; Kang et al. 1992) and pmms2::URA3 (*mms2::URA3*; Broomfield et al. 1998) were provided by W. Xiao (University of Saskatchewan). The plasmids p51::LEU2 (*rad51Δ::LEU2*), pDG347 (*rev3Δ::hisG-URA3-hisG*; Roche et al. 1994) and p18Δ1 (*rad18Δ::LEU2*; Fabre et al. 1989) were provided by F. Fabre (UMR217, CNRS). Each deletion/disruption cassette was released by appropriate digestions and was transformed into yeast.

The deletion cassettes of *RAD1*, *RAD10*, *RAD52* and *SRS2* were constructed using a PCR-based method to generate a DNA fragment consisting of the marker genes (*LEU2*, *URA3* and *HIS3MX6*) flanked by DNA from the 5′ and 3′ ends of the gene to be disrupted. For *rad1Δ::LEU2*, *rad10Δ::URA3* and *rad52Δ::URA3*, PCR using the plasmid YDp-L or YDp-U (Berben et al. 1991) as a template generated an intact *LEU2* or *URA3* gene flanked by sequences with homology to the *RAD1*, *RAD10* and *RAD52* loci, respectively. For *srs2Δ::HIS3MX6*, primers were used to amplify a 1.4-kb fragment with pFA6a-HIS3MX6 as a template (Wach et al. 1997). The PCR products were directly used to transform yeast.

The media used included rich yeast/peptone/dextrose medium (YPD) and synthetic complete medium (SC). SC lacking amino acids was used to score auxotrophies and to select transformants. SC medium lacking arginine and containing 60 mg of canavanine per liter was used to identify forward mutations in the *CAN1* gene. Routine culture and DNA manipulations followed standard protocols (Sambrook et al. 1989).

Table 1 *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source
FY1679C	*MATa/α ura3-52/ura3-52 leu2Δ1/leu2Δ1 his3Δ200/his3Δ200*	Huang et al. (1997)
FY1679-5C	*MATa ura3-52 leu2Δ1 his3Δ200*	B. Dujon Institut Pasteur
HD004	*MATa/α ura3-52/ura3-52 leu2Δ1/leu2Δ1 his3Δ200/his3Δ200 POL32/pol32Δ::kanMX4*	Huang et al. (1997)
FY1679-5CH	*MATa ura3-52 leu2Δ1 his3Δ200 pol32Δ::kanMX4*	Huang et al. (1999)
HA048	*MATa ura3-52 leu2Δ1 his3Δ200 rad1Δ::LEU2*	This study
HA049	*MATa ura3-52 leu2Δ1 his3Δ200 rad1Δ::LEU2 pol32Δ::kanMX4*	This study
HAL050	*MATα ura3-52 leu2Δ1 his3Δ200 rad10Δ::URA3*	This study
HAL051	*MATα ura3-52 leu2Δ1 his3Δ200 rad10Δ::URA3 pol32Δ::kanMX4*	This study
ORD5102-1A	*MATa ura3-52 leu2Δ1 rad51Δ::LEU2*	This study
HA026	*MATa ura3-52 leu2Δ1 rad51Δ::LEU2 pol32Δ::kanMX4*	This study
HA052	*MATa ura3-52 leu2Δ1 his3Δ200 rad52Δ::URA3*	This study
HA053	*MATa ura3-52 leu2Δ1 his3Δ200 rad52Δ::URA3 pol32Δ::kanMX4*	This study
HA038	*MATa ura3-52 leu2Δ1 his3Δ200 rad6Δ::LEU2*	This study
HA039	*MATa ura3-52 leu2Δ1 his3Δ200 rad6Δ::LEU2 pol32Δ::kanMX4*	This study
ORT3703	*MATa ura3-52 leu2Δ1 his3Δ200 rad18Δ::LEU2*	This study
HA031	*MATa ura3-52 leu2Δ1 his3Δ200 rad18Δ::LEU2 pol32Δ::kanMX4*	This study
ORT3700	*MATa ura3-52 leu2Δ1 trp1Δ63 rev3Δ::URA3*	This study
HA036	*MATa ura3-52 leu2Δ1 trp1Δ63 rev3Δ::URA3 pol32Δ::kanMX4*	This study
HA042	*MATa ura3-52 leu2Δ1 his3Δ200 mms2::URA3*	This study
HA044	*MATa ura3-52 leu2Δ1 his3Δ200 mms2::URA3 pol32Δ::kanMX4*	This study
HA019	*MATa ura3-52 leu2Δ1 his3Δ200 srs2Δ::HIS3MX6*	This study
HA023	*MATa ura3-52 leu2Δ1 his3Δ200 srs2Δ::HIS3MX6 pol32Δ::kanMX4*	This study
YTS7	*MATα ura3-52 leu2-3,-112 trp1-Δ can1 pol30-Δ1* + [*pBL230* (*POL30 TRP1*)]	Torres-Ramos et al. (1996)
HAL046	*MATα ura3-52 leu2-3,-112 trp1-Δ can1 pol32Δ::kanMX4 pol30-Δ1* + [*pBL230* (*POL30 TRP1*)]	This study
YTS31	*MATα ura3-52 leu2-3,-112 trp1-Δ can1 pol30-Δ1* + [*pBL230-922* (*pol30-46 TRP1*)]	Torres-Ramos et al. (1996)
HAL047	*MATα ura3-52 leu2-3,-112 trp1-Δ can1 pol32Δ::kanMX4 pol30-Δ1* + [*pBL230-922* (*pol30-46 TRP1*)]	This study

UV irradiation and methylation sensitivity

UV irradiation at 254 nm was delivered using a Stratalinker (Stratagene). Cells were grown overnight in YPD medium at 30 °C, diluted, plated on YPD (about 500 cells/plate) and irradiated with UV. After incubation in the dark at 30 °C for 3 days, the number of colonies was counted. UV survival was reported as the mean value for each UV dose from at least two independent experiments. The methyl methanesulfonate (MMS; Sigma) sensitivity assay was performed as follows. Cells were grown overnight in YPD medium at 30 °C and diluted to about 1×10^5 cells/ml. Appropriate dilutions of MMS were added to a 5-ml suspension of cells. Samples were incubated in YPD + 0.05% (or 0.01%) MMS. At several time points, aliquots were removed, mixed with an equal volume of freshly prepared 10% $Na_2S_2O_3$, diluted and plated on YPD. The number of colonies was counted after incubation at 30 °C for 3 days.

Two-hybrid screen

The two-hybrid screen was performed as previously described (Huang et al. 1999). Full-length *POL32* was fused in frame with the *GAL4* DNA-binding domain in the bait plasmid pASΔΔ. The FRYL two-hybrid library was a gift from M. Fromont-Racine and P. Legrain (Pasteur Institute, Paris). Yeast colonies that survived selection for histidine, leucine and tryptophane prototrophy and were blue in X-Gal filter assay were retained. Fragments from the library-derived plasmids (prey) in these positive colonies were amplified by PCR and sequenced.

Growth measurement and morphology analysis

To analyze growth characteristics on plates, haploid wild-type and mutant strains were first grown overnight in liquid YPD. The cells were then suspended in water, counted in a hemocytometer and diluted. Aliqots (5 μl) from each serial ten-fold dilution was spotted on solid YPD plates. Plates were incubated at 30 °C and/or 15 °C for various periods of time and photographed. For morphological analysis, aliquots from log phase cultures asynchronously grown at 30 °C or shifted to 15 °C were removed, fixed in 3.7% formaldehyde, sonicated and scored for bud size. Cells in which the bud was larger than 2/3 the size of mother cells were recorded as ''large-bud''. 4,6-Diamidino-2-phenylindole and immunofluorescence staining were performed as previously described (Huang et al. 1999).

Measurement of spontaneous mutation rate and UV-induced mutagenesis

Forward mutation to canavanine resistance was determined by fluctuation tests using the median method (Lea and Coulson 1948). All rates reported are the average of two independent experiments. In this analysis, independent 2-day-old colonies were inoculated into 5 ml of YPD liquid medium and grown non-selectively to $1–2 \times 10^8$ cells/ml. Cells were diluted to approximately 100 cells/ml in ten separate cultures for each strain, and again grown to $1–2 \times 10^8$ cells/ml in YPD. Cells were then harvested by centrifugation, washed once and resuspended in sterile water. Aliquots (100 μl) of appropriate dilutions were plated either onto canavanine-containing medium ($1–2 \times 10^7$ cells/plate) to identify forward mutations in *CAN1* or onto YPD to count viable cell numbers. Colonies appearing after 3–4 days of growth at 30 °C were counted. The number of Can^R colonies per 10^7 viable cells among the ten cultures was calculated and the median value from each set of ten cultures was used to determine the spontaneous mutation rate of a given strain. For UV-induced mutagenesis, the canavanine-containing SC-Arg plates (about 5×10^6 cells/plate) and YPD plates (about 500 cells/plate) were UV-irradiated at the indicated doses. The plates were incubated in the dark at 30 °C. Colonies on YPD plates and Can^R colonies on selection plates were counted after 4–5 days. The production of mutants was expressed as Can^R colonies per 10^7 viable cells for each UV dose tested.

HU-killing assay

Asynchronously growing log phase cultures were diluted to about 5×10^4 cells/ml in YPD + 0.2 M HU. Aliquots (10 µl) were plated onto YPD at timed intervals (0, 2, 4, 6 and 8 h). After incubation at 30 °C for 3 days, the number of colonies was counted.

Cell viability measurement

Asynchronously growing log phase cultures were diluted and plated on YPD (about 500 cells/plate). Triple plates were removed at defined time intervals after the shift to 15 °C and were incubated at 30 °C for 3 days. Colonies were scored and viability was expressed as a percentage, based on the number of colonies formed on plates without the shift to 15 °C.

Results

Genetic interaction of *pol32*Δ with mutations of the repair pathways

We examined UV and MMS sensitivity and also the spontaneous and UV-induced mutagenesis of *pol32*Δ in the FY1679 strain background. We observed that the *pol32*Δ mutation confers moderate sensitivity to killing by both UV and MMS (Figs. 1, 2). Concerning mutagenesis, measured by the canavanine resistance assay, the forward mutation rate of the *pol32*Δ mutant appears to be similar to or slightly lower than that of the wild-type strain, indicating that *pol32*Δ displays at the most a weak antimutator phenotype (Table 2). In contrast, UV-induced mutagenesis is affected. At UV irradiation doses producing no less than 50% viability (less than 50 J/m^2 and 15 J/m^2 for wild-type and *pol32*Δ mutant, respectively), the *pol32*Δ mutant displays a maximum mutation induction five-fold higher than the uninduced mutation frequency, versus a 20- to 100-fold increase in wild-type cells (Fig. 3). These results confirm the previous observations (Gerik et al. 1998) and indicate that Pol32 is strongly defective in UV-induced mutagenesis and very likely plays a role in PRR.

To ascertain in which pathway(s) the *POL32* gene belongs, epistasis analysis was performed by combining *pol32*Δ with *rad6*Δ and *rad18*Δ mutations (defects in PRR), *rad1*Δ and *rad10*Δ mutations (defects in NER), and *rad52*Δ and *rad51*Δ mutations (defects in recombinational repair). As shown in Fig. 1, the UV sensitivity of the *pol32*Δ *rad6*Δ double mutant equals that of the *rad6*Δ mutant (Fig. 1 A), indicating that *POL32* and *RAD6* belong to the same epistasis group. However, the UV sensitivity of the *pol32*Δ *rad18*Δ double mutant is slightly higher than that of the *rad18*Δ mutant (Fig. 1 B and further investigation below). Consistent with the notion of separable pathways, both the *pol32*Δ *rad1*Δ and *pol32*Δ *rad10*Δ double mutants, and the *pol32*Δ *rad52*Δ and *pol32*Δ *rad51*Δ double mutants exhibit a synergistic increase in UV sensitivity over that of the single mutants (data not shown), preventing assignment of *POL32* to the NER (*RAD3*) and recombinational repair (*RAD52*) pathways, respectively.

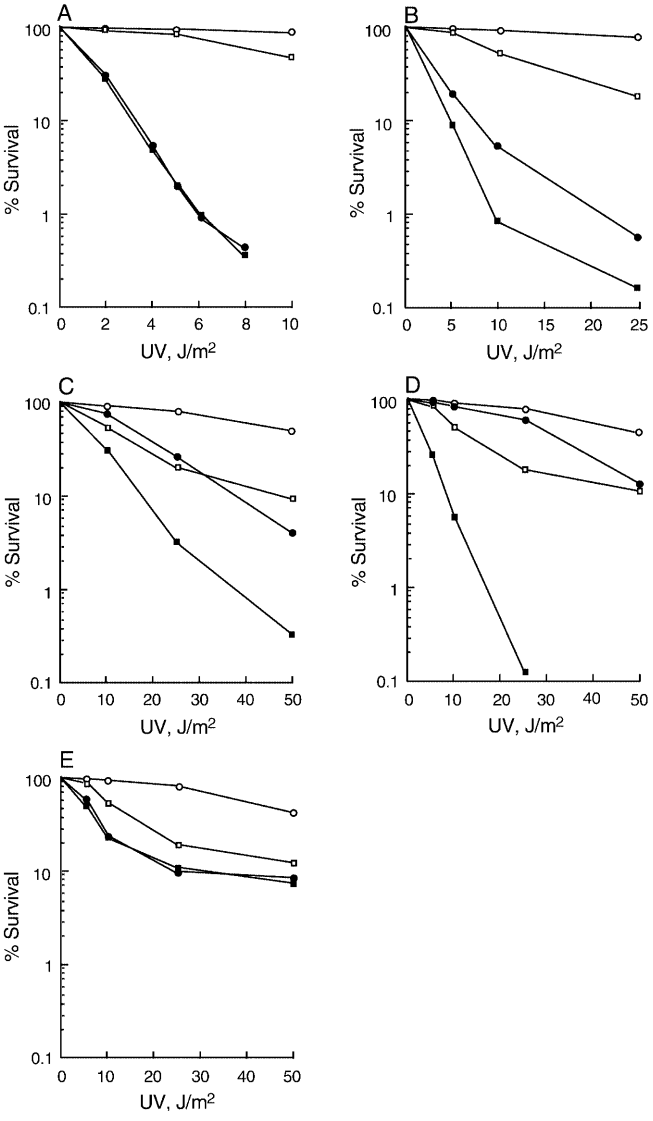

Fig. 1 A–E Ultraviolet radiation (*UV*) survival data for wild-type, *pol32*Δ and strains carrying mutations of *rad6*Δ, *rad18*Δ, *pol30–46*, *mms2* and *rev3*Δ. Survival curves represent an average of at least two different experiments for each strain. **A–E** ○ *POL32*, □ *pol32*Δ. **A** ● *rad6*Δ, ■ *pol32*Δ *rad6*Δ. **B** ● *rad18*Δ, ■ *pol32*Δ *rad18*Δ. **C** ○ *POL32* (YTS7), □ *pol32*Δ (HAL046), ● *pol30–46* (YTS31), ■ *pol32*Δ *pol30–46* (HAL047). **D** ● *mms2*, ■ *pol32*Δ *mms2*. **E** ● *rev3*Δ, ■ *pol32*Δ *rev3*Δ

Pol32 involvement in the *RAD6* mutagenic subpathway

Rad6 and Rad18 function in both the error-free and error-prone PRR, while some other proteins, such as PCNA (pol30–46), Mms2 and Rev3, are specific either to the error-free bypass or to the mutagenic pathway (Friedberg et al. 1995; Lawrence and Hinkle 1996; Torres-Ramos et al. 1996; Broomfield et al. 1998). To determine whether the Pol32 function could be assigned to either subset of the *RAD6* group, we compared the UV sensitivity of *pol32*Δ *pol30–46*, *pol32*Δ *mms2* and *pol32*Δ *rev3*Δ double mutants with that of the single mutants. The UV sensitivity of the *pol32*Δ *pol30–46* and

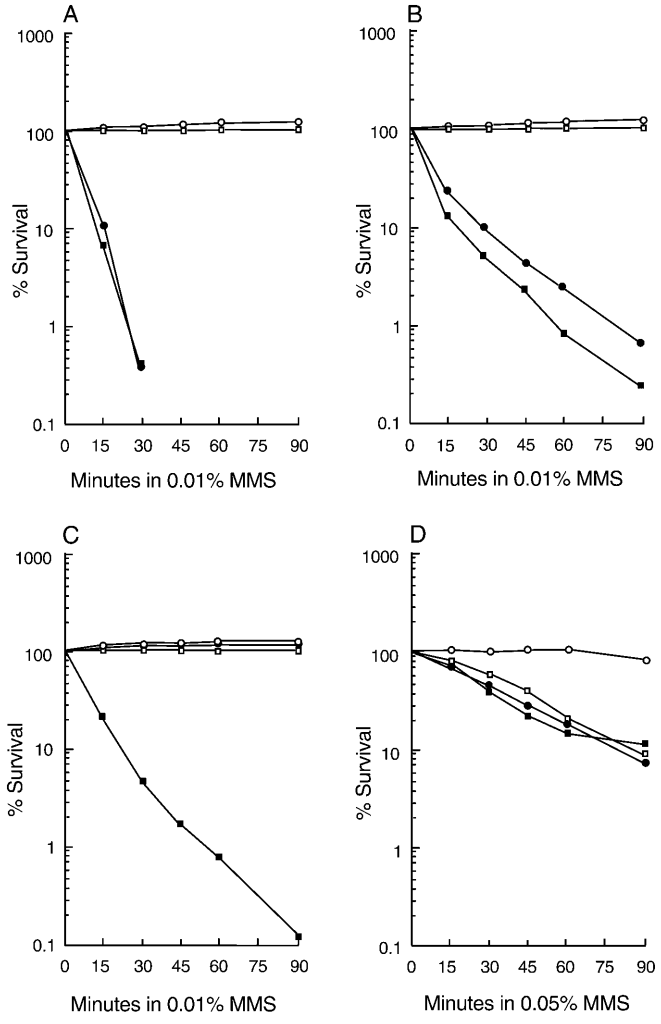

Fig. 2 A–D Methyl methanesulfonate (*MMS*) survival data for wild-type, *pol32*Δ and strains carrying mutations of *rad6*Δ, *rad18*Δ, *mms2* and *rev3*Δ. Survival curves represent an average of at least two different experiments for each strain. **A–D** ○ *POL32*, □ *pol32*Δ. **A** ● *rad6*Δ, ■ *pol32*Δ *rad6*Δ. **B** ● *rad18*Δ, ■ *pol32*Δ *rad18*Δ. **C** ● *mms2*, ■ *pol32*Δ *mms2*. **D** ● *rev3*Δ, ■ *pol32*Δ *rev3*Δ

*pol32*Δ *mms2* double mutants is greater than that of the single mutants (Fig. 1 C, D). In contrast, the UV sensitivity of the *pol32*Δ *rev3*Δ double mutant is comparable to that of the *rev3*Δ single mutant (Fig. 1 E). These results confirm that *POL32* belongs to the *RAD6* pathway, but further indicate that Pol32 is involved in the same mutagenic subpathway as Rev3.

Next, we examined the MMS sensitivity of the *pol32*Δ mutant in the presence of several mutations of the *RAD6* group genes. As previously observed, *rad6*Δ and *rad18*Δ single mutants are found to be highly sensitive to MMS. The *pol32*Δ *rad6*Δ double mutant has the same level of MMS sensitivity as the *rad6*Δ mutant, and the *pol32*Δ *rad18*Δ double mutant is slightly more sensitive to MMS than is the *rad18*Δ mutant (Fig. 2 A, B). However, the *pol32*Δ, *mms2* and *rev3*Δ single mutants are only moderately sensitive to MMS. This is not readily detectable in 0.01% MMS but becomes manifest in the presence of 0.05% MMS. The *pol32*Δ *mms2* double mutant is highly sensitive to MMS, comparable to an isogenic *rad18*Δ single mutant; and the effect of the two mutations is clearly synergistic (Fig. 2 C). In contrast, the *pol32*Δ *rev3*Δ double mutant is only moderately sensitive to

Table 2 Spontaneous mutation of *CAN1* in single and double mutants. Mutation rates represent the average of two independent experiments

Relevant genotype	Mutation rate ($\times 10^{-7}$)	Increase/decrease relative to *WT* (*x*-fold)
WT (*wild-type*)	5.1	1
*pol32*Δ	4.2	0.8
*rev3*Δ	2.9	0.6
*pol32*Δ *rev3*Δ	3.5	0.7
*rad6*Δ	11.1	2.2
*pol32*Δ *rad6*Δ	3.5	0.7
*rad18*Δ	15.0	3.0
*pol32*Δ *rad18*Δ	4.4	0.8
mms2	35.2	7.0
*pol32*Δ *mms2*	5.9	1.2
*rad1*Δ	13.6	2.7
*pol32*Δ *rad1*Δ	4.9	1.0
*rad10*Δ	9.0	1.8
*pol32*Δ *rad10*Δ	5.4	1.1
*rad52*Δ	46.0	9.0
*pol32*Δ *rad52*Δ	12.4	2.4
*rad51*Δ	41.4	8.1
*pol32*Δ *rad51*Δ	22.9	4.5

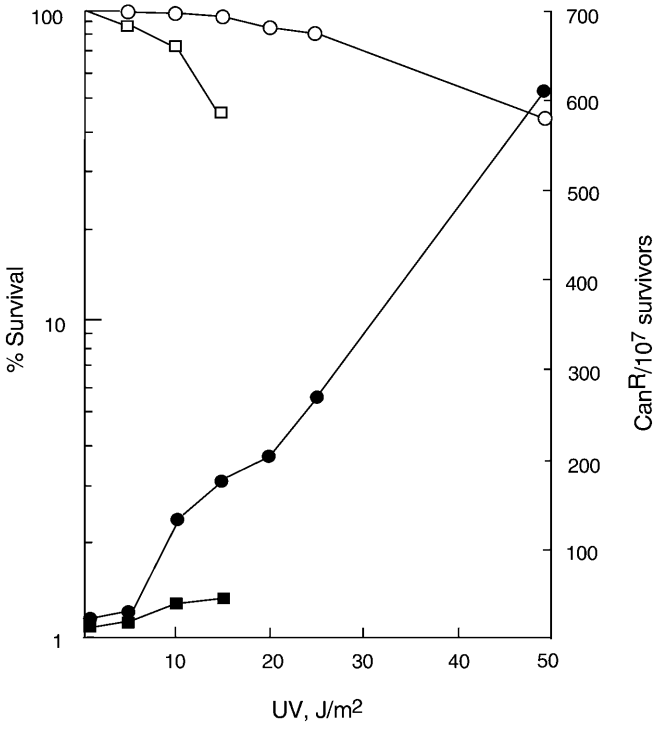

Fig. 3 UV-induced *CAN1* forward mutation of the wild-type strain and the *pol32*Δ mutant. A representative experiment is given in this figure. *Left ordinate*: UV survival data. ○ *POL32*, □ *pol32*Δ. *Right ordinate*: frequency of canavanine-resistant mutants per 10^7 survivors. ● *POL32*, ■ *pol32*Δ

MMS, but no more than the *pol32*Δ and *rev3*Δ single mutants (Fig. 2 D). These results confirm that *POL32* belongs to the error-prone subpathway.

POL32 dependence of *rad* mutator phenotypes

The *rev3* antimutator effect has been shown to decrease the magnitude of the mutator effect in *rad*1, *rad52*, *rad6*, *rad18* and *mms2* mutant cells (Roche et al. 1994, 1995; Broomfield et al. 1998; Xiao et al. 1999). To determine whether Pol32 is implicated in these phenotypes, we measured the spontaneous forward mutation rate at the *CAN1* gene by fluctuation tests in the wild-type, single and double mutants (Table 2). The mutation rate of the *pol32*Δ mutant is similar to or slightly lower than that of a wild-type strain. As expected, mutation in *REV3* reduces spontaneous mutagenesis, while mutation in *RAD6*, *RAD18*, *MMS2*, *RAD1*, *RAD10*, *RAD52* or *RAD51* causes an increase in the spontaneous mutation rate. The results of the comparison of the single and double mutants can be presented as follows. For *pol32*Δ *rad6*Δ, *pol32*Δ *rad18*Δ, *pol32*Δ *mms2*, *pol32*Δ *rad1*Δ and *pol32*Δ *rad10*Δ double mutants, spontaneous mutagenesis is reduced to the level of the *pol32*Δ single mutant or wild-type. In contrast, in double mutants *pol32*Δ *rad52*Δ and *pol32*Δ *rad51*Δ, spontaneous mutagenesis is reduced compared to *rad52*Δ and *rad51*Δ, but still much higher than that of the *pol32*Δ single mutant and wild-type. These observations indicate that *pol32*Δ shows an antimutator phenotype when combined to representative members of the three repair pathways of DNA damage response. However, the *rad6*Δ, *rad18*Δ, *mms2*, *rad1*Δ and *rad10*Δ mutator phenotypes depend completely or essentially on the function of *POL32*, while the *rad52*Δ and *rad51*Δ mutator phenotypes appear to be only partially *POL32*-dependent.

Protein–protein and genetic interactions of Pol32 and Srs2

SRS2 was originally identified as a suppressor of the UV sensitivity conferred by *rad6* and *rad18* mutations and was revealed to encode a DNA helicase with a 3′-to-5′ polarity (Aboussekhra et al. 1989; Schiestl et al. 1990; Rong and Klein 1993). The characterization of *srs2*-associated phenotypes and of functional suppressors of these phenotypes led to the proposal that the Srs2 helicase plays a complex and yet still poorly understood role in the choice of pathway for the repair of damaged DNA (Heude et al. 1995). Insights into the relation of Pol32 and Srs2 will now be described.

A two-hybrid screen was used to identify the protein(s) that interact(s) with the Pol32 protein. Of the His^+ and β-gal positive clones that contained an in-frame fusion between the *GAL4* activation domain and the yeast coding sequence, eight were found to correspond to gene *POL1* (Huang et al. 1999) and six to gene *SRS2* (Fig. 4). The fact that independent but overlapping clones were identified with the same bait plasmid is indicative of the specificity of this protein–protein interaction. The plasmid containing the shortest *SRS2* coding sequence and still displaying interaction is FR108 (residues 1132–1175 of the Srs2 sequence). This suggests that the interacting domain is located in the C-terminus of the Srs2 sequence.

The observed interaction between Pol32 and Srs2 in the two-hybrid system prompted us to examine their genetic interactions. For this purpose, a *pol32*Δ *srs2*Δ double mutant was constructed. Interaction was first investigated by analyzing the growth characteristics of cells containing single or double mutations. We noted that, at 30 °C, the plating efficiency of the *pol32*Δ *srs2*Δ double mutant is moderately decreased. The *pol32*Δ single mutant is cold-sensitive and the *srs2*Δ single mutant grows normally at 15 °C. In contrast, the *pol32*Δ *srs2*Δ double mutant is strongly sensitive to lower temperature. No clear growth was observed on YPD plates at 15 °C (Fig. 5 A, B). With the microscope, we observed that the cell cycle is arrested after several cell divisions, with only microcolonies being formed. At 30 °C and 15 °C, examination of the morphology of the cells indicated that the *POL32* deletion led to an increased proportion of large-budded cells relative to wild-type cells (Huang et al. 1999), and the *srs2*Δ mutant displays no particular cell-cycle phenotype. We observed that the proportion of large-budded cells in a *pol32*Δ *srs2*Δ strain is more marked than in either single mutant. These dumbbell-shaped cells have a single nucleus near or within the mother-bud neck and a short mitotic spindle (Fig. 5 C, D).

We further analyzed the viability of the *pol32*Δ *srs2*Δ mutant. Plates with single or double mutant cells were removed at defined time intervals after a temperature shift to 15 °C and were incubated at 30 °C for further growth. We observed that the viability of *pol32*Δ and *srs2*Δ single mutants is similar to that of wild-type. In

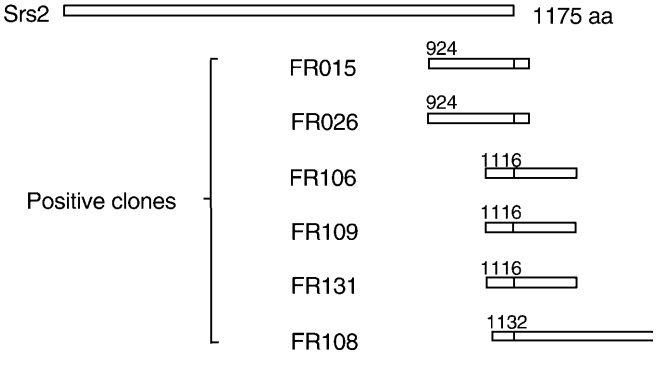

Fig. 4 Positive clones encoding Srs2 identified in the two-hybrid screens. The full-length yeast Srs2 protein is depicted *at the top*. *Bars* beneath represent the different fragments that were fused to the *GAL4* DNA-binding domain and participated in the positive interactions detected in two-hybrid screens. The *number* adjacent to the endpoints indicates the position of fusion relative to Srs2 amino acid sequence. It can be seen that the six clones correspond to three independent fusions

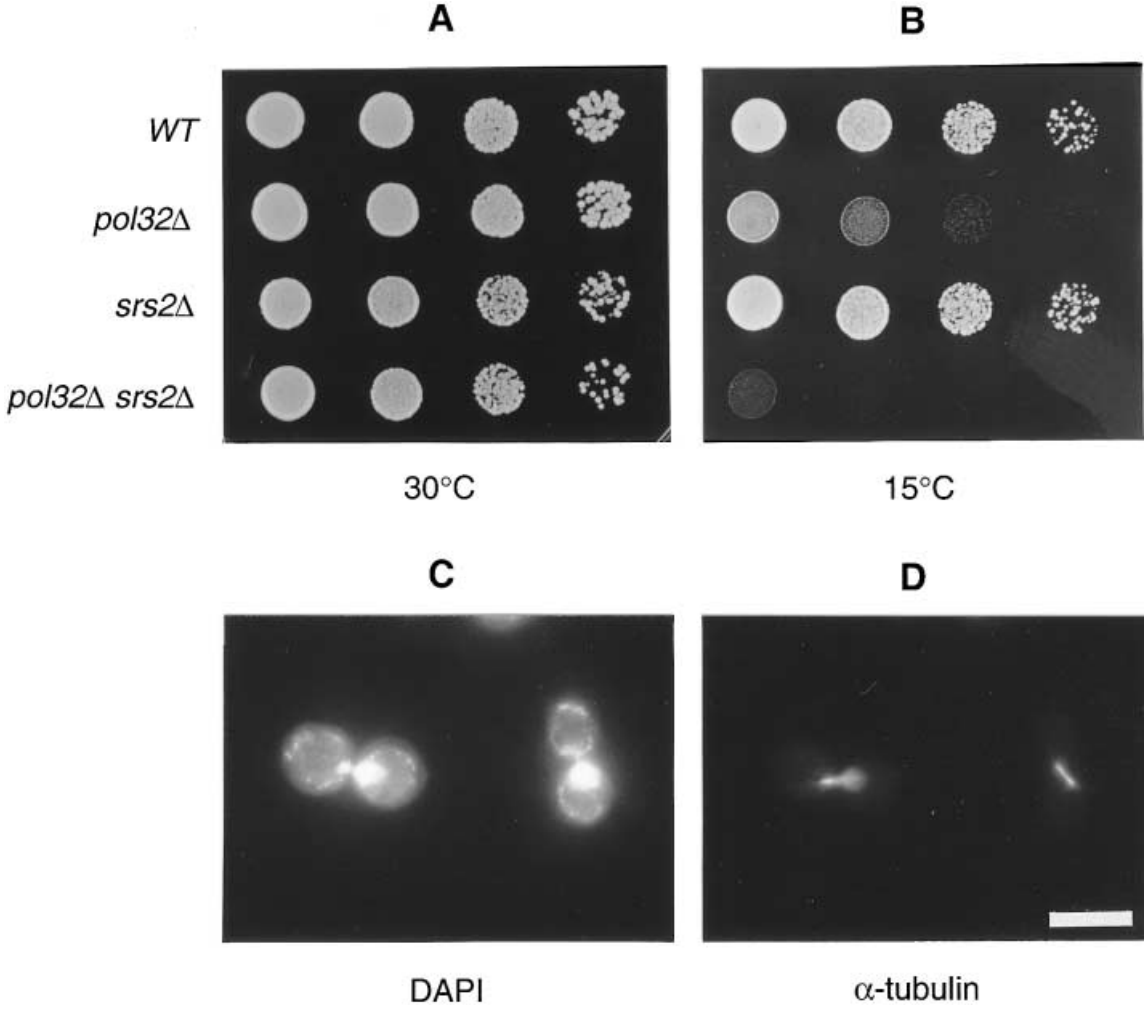

Fig. 5 A–D Characteristics of the *pol32*Δ *srs2*Δ double mutant. **A–B** Growth of the wild-type strain and isogenic mutants. Serial ten-fold dilutions of overnight cultures (1×10^6 cells/ml, 1×10^5 cells/ml, 1×10^4 cells/ml and 1×10^3 cells/ml) were prepared. An aliquot (5 μl) of each cell suspension was spotted onto solid YPD and grown at 30 °C for 3 days (**A**) or 15 °C for 10 days (**B**). **C–D** Morphology of *pol32*Δ *srs2*Δ double mutant cells at 15 °C revealed by 4,6-diamidino-2-phenylindole (*DAPI*, **C**) and anti-yeast α-tubulin antibody (**D**). The latter was visualized with a FITC-labeled goat anti-rat Ig secondary antibody. *Bar* 10 μm

contrast, after 24 h at 15 °C, approximately 70% of double mutant cells lose viability, with only about 10% of the cells surviving after 48 h at 15 °C (Fig. 6 A).

To determine whether synthetic lethality at low temperature results from an additive defect in DNA replication or in DNA repair caused by the *pol32*Δ and *srs2*Δ mutations, the relative sensitivities to HU, UV and MMS were determined for each mutant. The *pol32*Δ and the *srs2*Δ single mutations cause a modest sensitivity to HU, while the *pol32*Δ *srs2*Δ double mutant is much more sensitive than either single mutant (Fig. 6 B). In contrast, the double mutant has only a two-fold increased sensitivity to low-dose UV irradiation (5–10 J/m^2), as compared with each single mutant (Fig. 6 C). At higher doses, an inflexion of the curve is observed. Similarly, the double mutant has only a two-fold increased sensitivity to MMS as compared with each single mutant (Fig. 6 D). These results indicate that the double mutations may confer a more severe defect in DNA replication than does either single mutation.

Discussion

Contribution of Pol32 to DNA repair

Several lines of evidence from the present study support the view that the *POL32* gene belongs to the error-prone pathway within the *RAD6* group. First, *pol32*Δ is moderately sensitive to killing by both UV and MMS at a level characteristic of most PRR pathway mutants. This contrasts with mutants of the NER pathway, which are especially sensitive to UV and to chemicals that produce structurally distorting lesions, but are only marginally sensitive to MMS. It also contrasts with mutants of the recombinational repair pathway, which are extremely sensitive to ionizing radiation and MMS, but are less sensitive to UV as compared with mutants that affect the other two pathways (Friedberg et al. 1995). Second, *pol32*Δ displays an epistatic interaction with *rad6*Δ and *rev3*Δ, but displays an additive or synergistic interaction

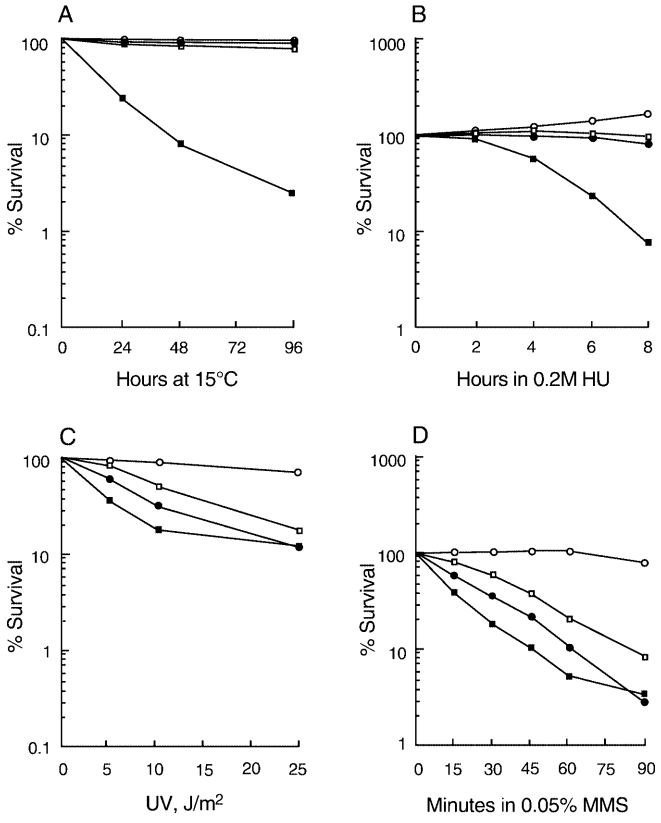

Fig. 6 A–D Viability, hydroxyurea (*HU*), UV and MMS survival data of the *pol32*Δ *srs2*Δ double mutant. **A** Viability of wild-type, *pol32*Δ, *srs2*Δ and *pol32*Δ *srs2*Δ cells at 15 °C, performed as described in Materials and methods. ○ *POL32*, □ *pol32*Δ, ● *srs2*Δ, ■ *pol32*Δ *srs2*Δ. **B** synergistic effect of *pol32*Δ and *srs2*Δ on HU sensitivity. ○ *POL32*, □ *pol32*Δ, ● *srs2*Δ, ○ *pol32*Δ *srs2*Δ. **C** UV survival data for wild-type, *pol32*Δ, *srs2*Δ and *pol32*Δ *srs2*Δ. ○ *POL32*, □ *pol32*Δ, ● *srs2*Δ, ■ *pol32*Δ *srs2*Δ. **D** MMS survival data for wild-type, *pol32*Δ, *srs2*Δ and *pol32*Δ *srs2*Δ. ○ *POL32*, □ *pol32*Δ, ● *srs2*Δ, ■ *pol32*Δ *srs2*Δ

with *pol30–46* and *mms2*, the two alleles involved in error-free PRR. Third, the absence of the gene *POL32* synergistically increases the UV sensitivity of *rad1*, *rad10*, *rad52* and *rad51* mutants, placing *POL32* outside of the NER and recombinational repair pathways. Finally and most convincingly, *pol32*Δ displays reduced UV-induced mutagenesis and the increase in the spontaneous forward mutation rate of different *rad* mutators relies entirely or partially on a functional *POL32* gene. Moreover, we observed that the *pol32*Δ mutation increases the UV and MMS sensitivity of the *rad18*Δ mutant, but not that of the *rad6*Δ mutant. As the *RAD6* and *RAD18* functions in both error-free and error-prone mechanisms and *rad6*Δ strains are more UV and MMS sensitive than are *rad18*Δ strains, this suggests that the *rad18*Δ mutations appear to block most, but not all, error-free or error-prone PRR. The simplest interpretation of our observation is that Pol32 functions in the error-prone pathway in a process distinct from that affected by *rad18* mutations.

Genetic and biochemical studies indicate that Rev3, the catalytic subunit of Pol ζ, plays a major role in the error-prone pathway (Lawrence and Hinkle 1996). The *rev3* mutations reduce both spontaneous and DNA damage-induced mutagenesis. This *rev3* antimutator effect has been shown to offset the magnitude of the mutator effect in *rad1*, *rad52*, *rad6*, *rad18* and *mms2* strains (Roche et al. 1994, 1995; Broomfield et al. 1998; Xiao et al. 1999). In experiments measuring spontaneous mutagenesis at a plasmid-borne *SUP4*-o locus, it has been observed that both *REV3*-dependent and -independent components contribute to the *rad1*, *rad6* and *rad18* mutator effects, while the *rad52* mutator effect is completely *REV3*-dependent (Roche et al. 1994, 1995). The *mms2* mutator effect on the *trp1-289* allele spontaneous reversion and the Can^R forward mutation largely depends on a functional *REV3* gene (Broomfield et al. 1998; Xiao et al. 1999). Our study reveals that the increase in the spontaneous Can^R forward mutation frequency of different *rad* mutators does not rely on *POL32* to the same extent. The *rad6*Δ, *rad18*Δ, *mms2*, *rad1*Δ and *rad10*Δ mutator effects rely entirely or largely on *POL32*, but the *rad52*Δ and *rad51*Δ mutator phenotypes are only partially *POL32*-dependent. Comparison of these results suggests that different forms of spontaneous lesions might be processed preferentially by *POL32*-dependent or *REV3*-dependent translesion synthesis. These two processes would be distinct but overlapping. In the future, a detailed determination (in isogenic strains) of the contribution of *POL32* and *REV3* to the magnitude and specificity (including the mutational spectrum) of different *rad* mutators from the three epistasis groups would help us to understand the mechanisms by which different forms of spontaneous DNA damage are processed.

The present study, which clearly places Pol32 in the error-prone pathway and reveals the dependence of *rad* mutators on a functional *POL32*, raises the question of the relation of Pol32 with PCNA, Pol3 and Rev3 and their respective biochemical function in translesion synthesis. Previous studies with the *pol30–46* and *pol3–13* mutations indicated an involvement of PCNA and Pol3 in the error-free and error-prone bypass of UV-damaged DNA, respectively (Torres-Ramos et al. 1996; Giot et al. 1997). PCNA is an essential processivity factor for DNA Polδ and interacts with Pol3 and Pol32 (Burgers 1998; Gerik et al. 1998). Therefore, it is possible that PCNA functions also in the mutagenic mode of damage bypass. PCNA interacts with various factors for distinct functions and Pol32 might be one of the protein components that links PCNA with mutagenesis. However, the involvement of Pol32 in mutagenic bypass may be through its association with the Pol3 and/or functions with Rev3, independently of Pol3. The mutagenic replisome is extremely complex indeed. The different polymerases may be specific to a given type of lesion and its sequence context or may play roles at different points in the cell cycle. This hypothesis is suggested by the observations that *REV3* is expressed at a constant low level throughout the cell cycle (Singhal et al. 1992), while the transcriptions of *POL3* and *POL32* are cell-cycle-

regulated and are induced in S-phase (Spellman et al. 1998; our unpublished data).

Pol32 and DNA helicase interaction

The characterization of *srs2*-associated phenotypes and of functional suppressors of these phenotypes led to the proposal that helicase Srs2 plays a complex role in the choice of the pathway for repairing damaged DNA. It is likely that the substrates of Srs2 are single-stranded gaps, which in the presence of Srs2 activity could be processed by the error-prone pathway, but in its absence are channeled into the recombinational repair pathway (Heude et al. 1995; Chanet et al. 1996). *SRS2* belongs to the *RAD6* group and is involved in mutagenesis (Friedberg et al. 1995; Liefshitz et al. 1998; Friedberg and Gerlach 1999). The *pol32*Δ *srs2*Δ double mutant shows only a slight increase in UV and MMS sensitivity in comparison with the *srs2*Δ single mutant, consistent with the notion that both genes belong to the error-prone PRR. Interestingly, we found that Pol32 and Srs2 interact specifically in the two-hybrid assay and that the *pol32*Δ *srs2*Δ double mutant seems to have a more severe defect in DNA replication than in repair, as manifested by an increase in the proportion of dumbbell-shaped cells, a decrease in viability and a large increase in HU sensitivity. *SRS2* is periodically transcribed during the cell cycle; and its expression rises shortly after bud emergence, a landmark of S-phase (Heude et al. 1995). This periodic pattern of expression and the presence of a degenerate *Mlu*I motif in its promoter region differentiate *SRS2* from the large majority of repair genes. Indeed, the presence of *Mlu*I motifs and periodic transcription at the G1/S boundary characterize many genes involved in DNA replication, including *POL1*, *POL2*, *POL3*, *PRI1*, *PRI2* and *POL32*, to name only a few (McIntosh 1993; Huang et al. 1999). *POL32* is transcribed during the cell cycle with a peak of transcription at the G1/S boundary (Spellman et al. 1998; our unpublished data). It is therefore likely that *SRS2* is cell-cycle-regulated in coordination with a number of genes needed for DNA synthesis, such as *POL32*, and also with a few genes involved in repair, such as *RAD5*, *RAD51* and *RAD54* (Spellman et al. 1998). The similar cell cycle control of *SRS2* and *POL32*, the involvement of both proteins in the mutagenic repair pathway, the interaction of Pol32 and Srs2 in the two-hybrid system and their genetic interaction suggest that Pol32 and Srs2 play a particular role in S-phase, probably a repair function associated with replication.

The functional significance of the Pol32/Srs2 interaction in DNA replication and repair remains to be elucidated. However, the recent findings of Lee et al. (1999) and Kamath-Loeb et al. (2000) may help us understand the basis of some phenotypes revealed in the *pol32*Δ *srs2*Δ double mutant. It is demonstrated that Srs2 and Sgs1 proteins play redundant roles, because the effects on rRNA transcription and DNA replication are not observed in the *srs2*Δ or *sgs1*Δ single mutants, but only in the *srs2*Δ *sgs1-ts* double mutant at the non-permissive temperature (Lee et al. 1999). Simultaneous deletion of *SRS2* and *SGS1* is lethal. Sgs1 is a member of the RecQ family of DNA helicase and shares homology with the human BLM (the product of the Bloom's syndrome gene) and WRN (the product of Werner's syndrome gene; Karow et al. 2000). Interestingly, WRN functionally interacts with Polδ; and WRN-mediated stimulation of Polδ activity in vitro requires the Pol32 of *S. cerevisiae* (Kamath-Loeb et al. 2000). Therefore, it is conceivable that the association of the Pol32 polypeptide with the catalytic subunit(s) of one or several DNA polymerases plays an interactive role in recruiting the Srs2 DNA helicase and possibly also other DNA helicases of the RecQ family, according to the biological circumstances when the polymerase needs to move the replication fork or needs to bypass spontaneous or induced DNA damages. This proposed role of the Pol32 polypeptide in protein–protein interaction may also explain the limited sequence identity observed between Pol32 and *Schizosaccharomyces pombe* Cdc27, a subunit of the *S. pombe* Polδ (Gerik et al. 1998), and the p66 subunit of mammalian Polδ (Hughes et al. 1999). In fact, these subunits may have evolved to interact with various members of the RecQ family of DNA helicases, whose sequence is strongly conserved but only within the central helicase region (Karow et al. 2000).

Acknowledgements We thank F. Fabre (UMR217 CNRS), W. Xiao (University of Saskatchewan), L. Prakash (University of Texas Medical Branch), G. Berben (Centre de Recherches Agronomiques, Gembloux) and P. Philippsen (Universitat Basel), for the gift of disruption cassettes and mutant strains; we thank M. Fromont-Racine and P. Legrain (Institut Pasteur) for the FRYL yeast two-hybrid library; and we thank C. Schwintner and S. Loeillet (Institut Curie, UMR144 CNRS) for the construction of *rad51*Δ, *rad18*Δ and *rev3*Δ strains. We also thank M. Philippe and J.-C. Chuat (UPR41 CNRS) for valuable comments on the manuscript and K. Smith (Institut Curie, UMR144 CNRS) for English correction. This work was supported by the Centre National de la Recherche Scientifique (CNRS, France).

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