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This paper is dedicated to the memory of Professor Wacław Gajewski

The essential DNA polymerases δ and ε are involved in repair of UV-damaged DNA in the yeast *Saccharomyces cerevisiae*★

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We have studied the ability of yeast DNA polymerases to carry out repair of lesions caused by UV irradiation in *Saccharomyces cerevisiae*. By the analysis of postirradiation relative molecular mass changes in cellular DNA of different DNA polymerases mutant strains, it was established that mutations in DNA polymerases δ and ε showed accumulation of single-strand breaks indicating defective repair. Mutations in other DNA polymerase genes exhibited no defects in DNA repair. Thus, the data obtained suggest that DNA polymerases δ and ε are both necessary for DNA replication and for repair of lesions caused by UV irradiation. The results are discussed in the light of current concepts concerning the specifity of DNA polymerases in DNA repair.

The repair of damaged or inappropriate bases in DNA by enzyme-catalysed excision is common to all living organisms. Depending on whether the damaged bases are excised as free entities or as components of oligonucleotide fragments, these processes are designated base excision repair (BER) or nucleotide excision repair (NER) systems. Both systems have widely divergent mechanisms and create gaps of different sizes in single-

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Abbreviations: BER, base excision repair; MMS, methyl methanesulfonate; NER, nucleotide excision repair.

stranded DNA (for a review see Budd & Campbell, 1997), but they share one common step. The gaps formed during excision are always filled in by DNA polymerases during DNA repair synthesis. Although the total extent of DNA synthesis during repair is small compared to the amount of chromosomal replication, the cellular DNA polymerases must be able to find all the incised lesions and repair them accurately enough for cell survival. Despite the ubiquitous requirement for DNA synthesis, the involvement of particular DNA polymerases in repair is still controversial and not completely understood.

Genetic and biochemical studies have identified several nuclear DNA polymerases in yeast and in higher eucaryotic cells (Campbell & Newlon, 1991; Sugino, 1995; Budd & Campbell, 1997; Wang, 1991). Three different DNA polymerases are essential and are responsible for chromosomal replication (Budd & Campbell, 1989; 1993; Boulet *et al.,* 1989; Budd *et al.,* 1989, Morrison *et al.,* 1990; Araki *et al.,* 1992). These are DNA polymerases α, ε and δ. DNA polymerase α, encoded by the gene *POL1,* is physically associated with DNA primase and is belived to extend RNA primers at origins of replication and during lagging-strand replication. DNA polymerases ε and δ, encoded by the genes *POL2* and *POL3* respectively, are catalytically similar multiprotein complexes and have properties expected for DNA elongation, although there is still insufficient information to assign specific roles to each of them *in vivo* (Budd & Campbell, 1993; 1997). Other DNA polymerases are not essential. These are DNA polymerase β encoded by the gene *POL4* (Prasad *et al.,* 1993; Budd & Campbell, 1997) and DNA polymerase ζ encoded by the genes *REV3* and *REV7* (Morrison *et al.,* 1989; Nelson *et al.,* 1996). Recently a new DNA polymerase (polymerase V) was discovered in the yeast genome as an open reading frame that encodes a polypeptide with some homology to the α-type DNA polymerases (Sugino, 1995). It should be noted that mitochondria have specific DNA polymerase γ, encoded by the gene *MIP1,* which is probably responsible for both replication and repair of mitochondrial DNA (Foury, 1989).

The role of polymerase β in yeast is unknown. In mammalian cells DNA polymerase β is responsible for the repair of short gaps formed during BER and may be involved in the repair of DNA double strand breaks. In contrast, yeast strains carrying a deletion of the *POL4* gene are viable and show no sensitivity toward UV, X-rays or methyl methanesulfonate (MMS), suggesting that DNA polymerase β plays no role in DNA replication, nucleotide or base excision repair and in double strand break repair (Budd & Campbell, 1997). Similarly, no metabolic effect was observed in strains carrying a deletion of polymerase V gene (Sugino, 1995). Only the role of DNA polymerse ζ in repair is better understood. From the studies of Lawrence and his group it is evident that this polymerase functions in trans-lesion DNA synthesis (Morrison *et al.,* 1989; Nelson *et al.,* 1996). *Rev3* deletion mutants exhibit also moderately increased sensitivity toward UV and MMS treatment. Recently it was found that DNA polymerase ζ cooperates closely with DNA polymerase δ in the repair of lesions induced in yeast DNA by MMS (Hałas *et al.,* 1997) and in mismatch repair (Longley *et al.,* 1997). However, the *in vitro* studies of Wang *et al.* (1993) showed that this polymerase does not participate in UV-light lesion repair. These results were confirmed later by Budd & Campbell (1995) and the reason of the observed sensitivity of *rev3*Δ mutants toward UV-light is not clarified. Therefore, DNA polymerase ζ mutant was included in these investigations.

Studies on the involvement of the essential DNA polymerases in different DNA repair systems showed that DNA polymerase α does not appear to perform a significant role,in nucleotide or base excision repair systems in either yeast or mammalian cells. However, in cells with extremly efficient repair system, e.g. in *Xenopus* oocytes, Pol α antibody inhib-

its repair synthesis of UV-irradiated nuclear extracts (Oda *et al.*, 1996). Perhaps the necessity of high rate repair requires all of DNA polymerases present in the cell which potentially are capable of performing repair synthesis. In contrast, DNA polymerases ε and δ clearly play an important role in excision repair pathways. Studies *in vivo* with *S. cerevisiae* DNA polymerase mutants showed that Pol ε and especially Pol δ are actively involved in repairing breaks induced by a broad spectrum of mutagens. It is known from our investigations and those of others that DNA polymerase δ is required for the repair of damages caused by exogenous DNA methylation, *via* BER and other systems (Suszek *et al.*, 1993; Blank *et al.*, 1994; Hałas *et al.*, 1997), and for the repair of UV-induced damages, chiefly *via* NER (Budd & Campbell, 1995). This polymerase is also required in post-replicational repair of UV-damaged DNA (Torres-Ramos *et al.*, 1997) and in mismatch repair (Oda *et al.*, 1996). DNA polymerase ε seems to be involved together with DNA polymerase δ in the repair of UV-damaged DNA, where both are equally active and, according to Budd & Campbell (1995), may substitute each other. In post-replicational repair and in MMS-induced lesions repair this polymerase plays a minor, if any, role. On the other hand, Wang *et al.* (1993) showed in their *in vitro* studies with the use of soluble yeast repair extracts that DNA polymerase ε has a preference for repair of UV and osmium tetroxide lesions repaired mostly by BER. Other essential DNA polymerases only influence repair synthesis catalysed by Pol ε indirectly, but cannot substitute for it. Having this in mind, in this report we present the results of an experimental approach that utilises temperature sensitive mutants of DNA polymerases δ and ε, in addition to the deletion mutant *rev3*Δ of DNA polymerase ζ, for *in vivo* studies of their influence on the repair of UV induced lesions. We found, in accordance with Budd & Campbell (1995), that both essential DNA polymerases are involved in the repair of these lesions and we also found that both enzymes are necessary for performing such repair. DNA polymerase ζ, in contrast to the repair of MMS-induced lesions, is not involved in the NER system.

MATERIALS AND METHODS

Strains and media. The *S. cerevisiae* strains used in these experiments were as follows:

- **HB75** a/α *trp1/trp1 met/MET ura1/URA his7/his7 leu2/LEU pol3-1/pol3-1*
- **HB75/1** a/α *trp1/trp1 met/MET ura1/URA his7/his7 leu2/LEU*
- **HB89** a/α ade2/ADE ade5/ADE ura3-52/URA leu2/LEU pol2-18/pol2-18
- **AMY32** *α rev3Δ::LEU2 arg4-17 leu2-3,112 his3-Δ1 trp1 ura3-52*
- **CL1265-7C** *α REV3 arg4-17 leu2-3,112 his3-Δ1 trp1 ura3-52*
- **HB107** *a/α trp1/trp1 arg4-17/arg4-17 leu2-3,112/LEU his3-Δ1/HIS ura3-52/URA rev3^0/rev3^0*
- **YPR001** a *leu2-3-112 trp1-298 ura3-52 pep4::URA3 pol4Δ::LEU2*
- **CB001** a *leu2-3-112 trp1-298 ura3-52 pep4::URA3*

Temperature-sensitive *pol3-1* mutants were from Dr. C. Newlon and Dr. L. Hartwell (New Jersey Medical School, Newark and University of Washington, Seattle respectively); the *pol2-18* mutant was from Dr. A. Morrison (National Institutes of Health, North Carolina); the *rev3*Δ mutant was from Dr. C. Lawrence (University of Rochester, Rochester N.Y.). Diploids and multiple mutants were constructed in our laboratory by standard techniques of yeast genetics (Sherman *et al.*, 1971). Other mutants were from the Yeast Genetics Stock Center (University of California, Berkeley). Standard solid and liquid complete media (YPD) and minimal medium (GO) were as described earlier (Baranowska *et al.*, 1985). The viability of cells was determined by incubation of appropriate dilutions of cells

on YPD plates at 23°C (for temperature-sensitive strains) or 30°C (for other strains).

Labelling, UV-irradiation and repair incubation. Labelling of cells was as described earlier (Hałas *et al.,* 1997). Labelled cells suspension (5 ml) was centrifuged and washed twice with 0.1 M phosphate buffer (pH 7.0). Cells were resuspended in 5 ml of the same buffer, chilled in an ice bath and irradiated with constant stirring by a Philips mercury lamp at a dose rate 1.5 $J/m^{-2}s$ in open petri dishes. After irradiation cells were washed once with YPD and incubated further for 6 h in the same medium or spheroplasted immediately. All operations were performed in the dark to avoid photoreactivation.

Spheroplast formation and alkaline sucrose gradient sedimentation. After incubation, cells were collected, placed on ice, washed with ice-cold 0.1 M phosphate buffer, pH 7.0, and with 0.05 M EDTA, pH 7.5, resuspended in 0.1 M Tris/HCl pH 9.0, 0.05 M EDTA, 2% β-mercaptoethanol, and incubated on ice for 30 min. Cells were then centrifuged, resuspended in 1 ml of 1 M sorbitol, 0.1 M EDTA, pH 7.5, with 1 mg/ml of Zymolase 100T (Seikagaku, Corp. Tokyo, Japan) and incubated for 10 min at 37°C. Spheroplasts were centrifuged at 1000 r.p.m. and gently resuspended in 0.15 M NaCl, 0.1 M EDTA, pH 8.0, at a concentration of 1×10^8 spheroplasts/ ml. Spheroplasts suspension (0.1 ml) was mixed with UV-endonuclease from *Micrococus luteus* (12 μl per sample, obtained as described by Resnick *et al.* (1983) and layered immediately onto a 0.2 ml separating layer of 15% sucrose dissolved in 0.01 M Tris, 0.01 M EDTA, (pH 8.0), 1 M NaCl, followed by 50 μl of 10% Nonidet P-40 in 0.1 M phosphate buffer, pH 7.0, placed on top of 5 ml of 15–30% sucrose gradient, prepared in a solution of 0.3 M NaOH, 0.7 M NaCl, 0.03 M EDTA, 0.1% sarcosyl, and followed by 5 μl of ^{3}H-labelled λ phage DNA solution, used as a sedimentation marker. Gradients were centrifuged in an SW 50.1 rotor (Beckman) at 4°C and 13000 r.p.m. for 18 h. Fractions (0.2 ml) were collected from the bottom of the tube and radioactivity was determined according to the method described earlier (Hałas *et al.,* 1997). The relative average molecular mass of DNA in a fraction (M_n) was calculated according to Freifelder (1970) by comparing the distance sedimented by yeast DNA (d_2) with the distance sedimented by λ DNA (d_1), with the use of equation $(d_1/d_2) = (M_1/M_2)^{0.41}$. Total radioactivity used in each gradient ranged from 7000 to 9000 c.p.m. and the recovery was over 90%.

RESULTS

It is important for the present studies to have DNA polymerase mutants with severe defects since it is likely that only a small amount of polymerase activity is required for DNA repair. As previously demonstrated, the temperature sensitive '*pol3-1* mutant of DNA polymerase δ synthesises little chromosomal DNA at the restrictive temperature *in vivo* (Suszek *et al.,* 1993; Hałas *et al.,* 1997). Similar results were obtained for the mutant *pol2-18* or *pol2-12* of DNA polymerase ε (Hałas *et al.,* 1997). The results obtained are consistent with those demonstrated earlier by Araki *et al.* (1992) and by others (Budd & Campbell, 1993; 1995; Budd *et al.,* 1989; Wang *et al.,* 1993). It should be noted, however, that at the permissive temperature none of these mutants are UV-sensitive or MMS-sensitive (Budd & Campbell, 1995; Hałas *et al.,* 1997). The mutant strain defective in nonessential DNA polymerase ζ has a deletion in the catalytic region of the *REV3* gene and almost certainly lacks the *in vivo* function of the corresponding polymerase. As can be seen from Fig. 1, deletion of the *REV3* gene in the tested strain results in an increase of its sensitivity toward UV and in a reduction of UV-induced reversion of the *arg4-17* mutation, which is characteristic for the *rev3* mutant described earlier by Lawrence & Christensen (1981).

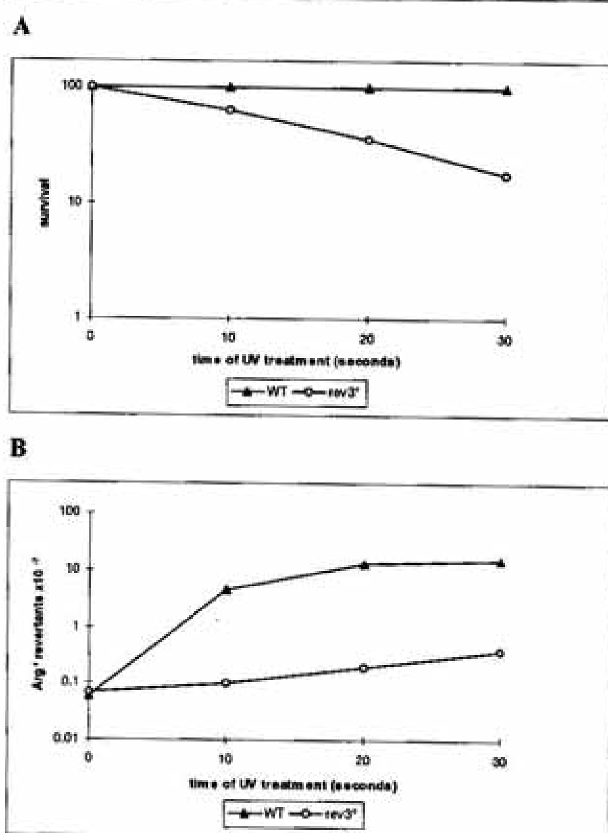

Figure 1. Comparison of the wild-type strain CL1265-7C and Δ*rev3* mutant strain AMY32 in: (A), survival of UV-irradiation and: (B), UV-induced reversion of *arg4-17* mutation.

Cell survival was determined on YPD agar plates after four days growth at 30°C. The frequency of reversion was tested on minimal medium GO supplemented with appropriate nutritional additions but without arginine. The number of revertants in the *arg* locus was scored after 7 days of incubation.

Involvement of DNA polymerases δ and ε in the repair of UV-irradiated DNA

To demonstrate the requirement of a given DNA polymerase in DNA repair we analysed relative molecular mass changes of DNA from cells irradiated with UV only and from cells incubated in YPD after irradiation to allow repair. Such analysis is based on the assumption that DNA polymerase mutants defective in a repair function will be proficient in the excision of damaged bases but deficient in filling in and closing the resulting gaps. Thus, a polymerase mutant would be expected to accumulate single-stranded breaks in its chromosomal DNA following UV-irradiation in a similar way as DNA ligase mutants (Wilcox & Prakash, 1981). For minimizing gap formation resulting from defective replication, cells of temperature sensitive mutants were grown at the permissive temperature prior to irradiation. Since after treatment cells were incubated at the restrictive temperature we were assured that such incubation has no influence on the size of cellular DNA (Hałas *et al.*, 1997). Thus, the changes observed in the DNA peak position represented only DNA breakage after irradiation or its repair but not the changes caused by defective replication. DNA was labelled by growing cells with [^{14}C]adenine overnight. The cells were harvested and treated with a UV dose of 45 J/m^2. Repair was allowed to occur in YPD medium at permissive (28°C) and restrictive (38°C) temperature. Cells were then harvested and the size of DNA was determined on an alkaline sucrose gradient with λ DNA as relative molecular mass marker.

Typical sedimentation profiles of DNA from cells irradiated with UV and allowed to undergo repair are presented in Fig. 2. DNA from nonirradiated cells, regardless of whether from the mutant or wild-type strain, sedimented similarly in the lower half of the gradient at the average peak position corresponding to 4–5 × 10^8 M_n even though the total shape of the curve differ from each other (A, B, C, solid lines). Irradiation of the cells resulted in a different pattern of DNA sedimentation. Most of the DNA sedimented now at the upper part of the gradient, reaching the peak value equal to M_n 5–8 × 10^7 (A, B, C, open triangles). During 6 h of incubation at 28°C the DNA peak shifted again to the position of chromosomal size DNA (M_n 3–4 × 10^8, D, E, F, closed circles). Therefore, the capacity of DNA repair at the permissive temperature 28°C was similar in the *pol2-12* or *pol3-1* mutant strains and in the wild type strain. But when the cells of UV-irradiated mutant strains were incubated at the restric-

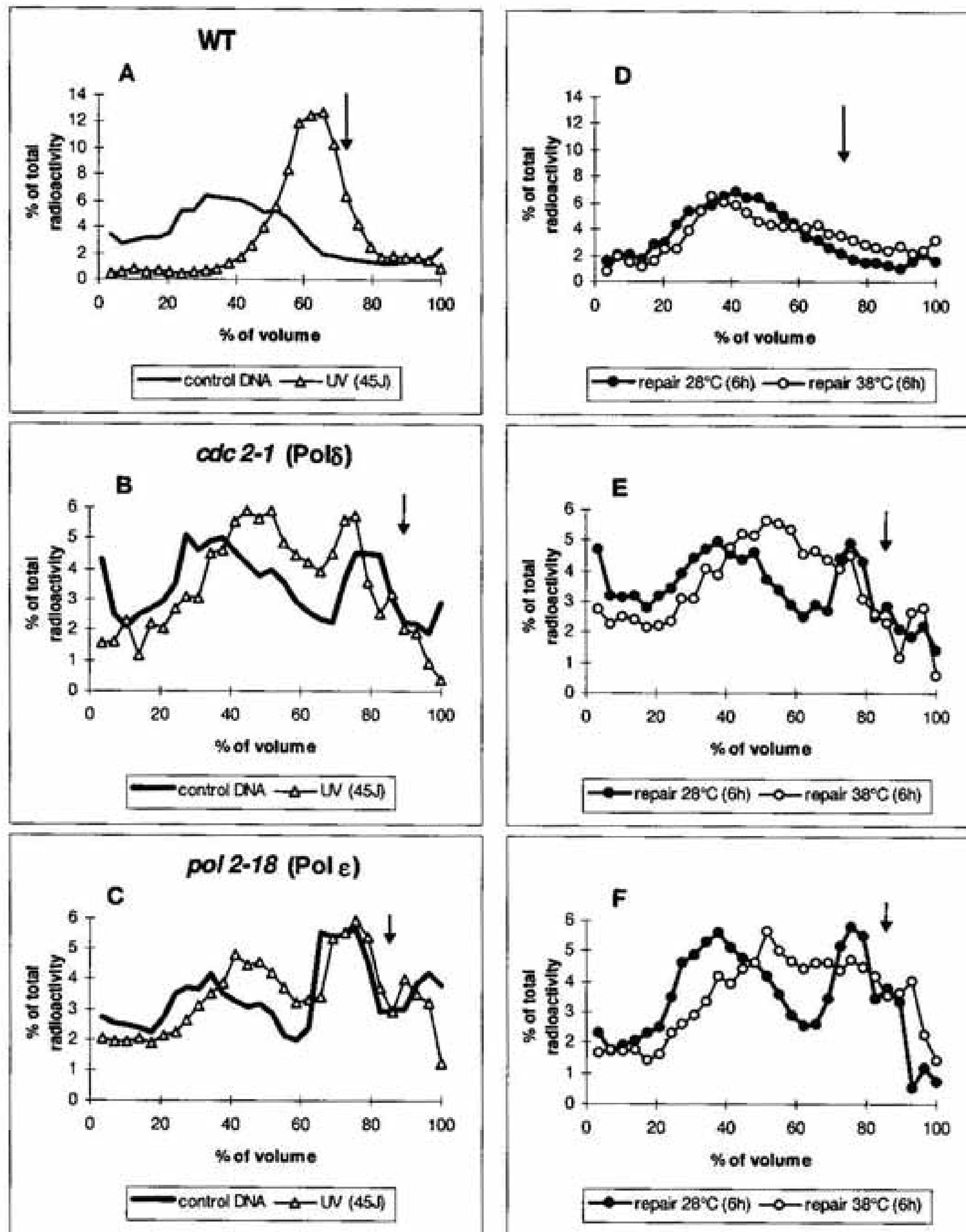

Figure 2. Alkaline sucrose gradient sedimentation analysis of UV-induced single strand break repair in DNA from: HB75/1, the wild-type strain, HB75, the *pol3-1* mutant strain in the gene of DNA polymerase δ, and HB89, the *pol2-18* mutant strain in the gene of DNA polymerase ε.

Effects of UV-irradiation on DNA from : (A) wild-type strain; (B) *pol3-1* mutant strain, and (C) *pol2-18* mutant strain. (–) DNA from unirradiated cells, control; (Δ) DNA from cells irradiated with UV but not allowed further incubation repair. Effects of 6 h incubation repair in restrictive temperature 38°C (○), or permissive temperature 28°C (●) from: **D**, wild-type strain; **E**, *pol3-1* strain and **F**, *pol2-18* strain. The arrow points to the sedimentation position of λ DNA.

tive temperature 38°C the difference in the repair capacity of mutant strains appeared clearly. After 6 h of repair incubation, only in the wild-type strain the average peak position of DNA shifted toward the position of unirradiated DNA, reaching a value of M_n equal to 3.5×10^8 – almost identical with the control position of unirradiated DNA (Fig. 2, D). In

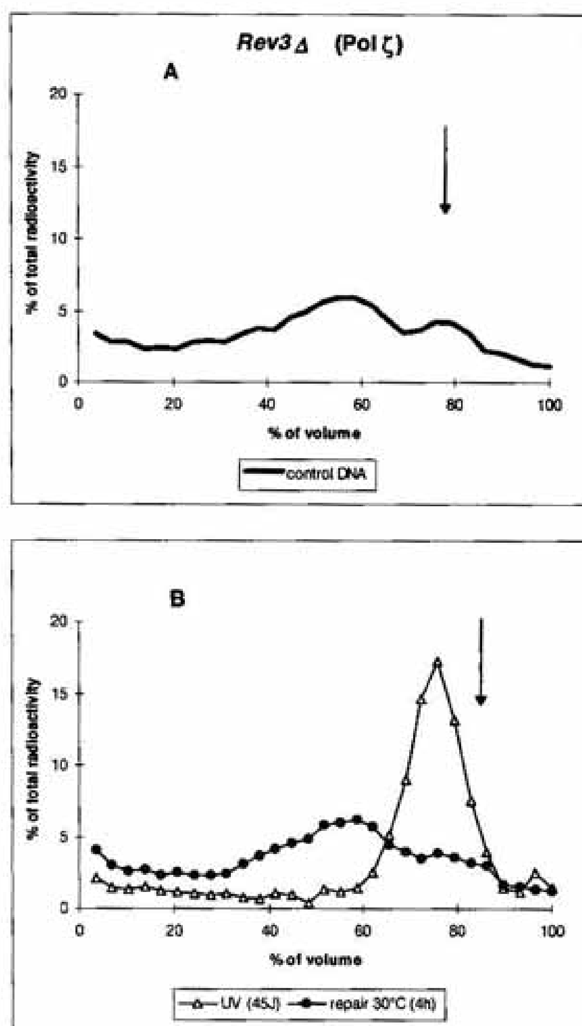

Figure 3. Repair of single strand breaks in HB 107 diploid strain containing a deletion in DNA polymerase ζ.

Cells were irradiated with 45 J/m^2, the same dose as other strains presented in Fig. 2, and allowed to undergo repair incubation for 4 h at 30°C. A (–) untreated DNA, control; B (▽) DNA treated with UV; (●) UV-treated DNA after repair-incubation.

the *pol3-1* and *pol2-12* mutants the observed increase in DNA relative molecular mass after 6 h incubation was very limited, reaching average M_n values equal to $5-10 \times 10^7$, similar for both tested strains (Fig. 2 E, F, open circles). Thus, the efficiency of repair is drastically reduced in these mutants when compared to the wild type strain. Since this reduction appeared in each of the single polymerase mutants, it seems that both DNA polymerase δ and ε play a specific and important role in gap filling during repair of UV-induced lesions.

Mutation in DNA polymerase ζ does not give rise to a repair defect

The nonessential DNA polymerase ζ seems not to be involved in the repair of lesions caused by UV-irradiation. Figure 3 illustrates data obtained for the strain carrying a deletion in the *rev3* gene which encodes the catalytic subunit of DNA polymerase ζ. Panel A illustrates the sedimentation profile of control, non irradiated mutant cells. The average value of M_n equal to $3-4 \times 10^8$ is similar to that observed in the wild-type cells (see Fig. 2). Panel B represents curves obtained for irradiated cells and for cells allowed to undergo 4 h repair incubation at 30°C. The dose of UV was 45 J/m^2, same as in the case of *pol3-1* or *pol2-18* mutants. After exposure to UV light, DNA sediments in the upper half of the gradient and represents only broken chromosomes. After repair incubation for 4 h DNA peak shifted to the position of undegraded DNA (M_n $2-4 \times 10^8$) and the sedimentation pattern was almost indistinguishable from that of unirradiated DNA (panel A). Thus, the inactivation of DNA polymerase ζ did not change the capacity of the mutant strain for repair of lesions induced by UV-light. Similar results was also obtained for the *pol4Δ* mutant carrying a deletion in the *POL4* gene of DNA polymerase β_{70} (results not shown).

DISCUSSION

Using as an assay for DNA repair the persistence of transient breaks after UV-irradiation in yeast polymerase mutants we present here *in vivo* evidence that in *S. cerevisiae* two replicative DNA polymerases δ and ε are required for the repair of gaps formed in DNA after pyrimidine dimer removal. Our conclusions are consistent with the finding of Budd & Campbell (1995). The only difference between our

observations and those of Budd and Campbell concerns the activities of single DNA polymerase mutants. We found that both DNA polymerases δ and ε seem to be indispensable in repair synthesis, since strand breaks formed in DNA after pyrimidine dimer removal persist equally well in *pol3-1* or *pol2-18* mutants when incubated under restrictive conditions. According to Budd & Campbell (1995) the persistence of breaks appeared only in double mutants *pol3-1 pol2-12* (or *pol2--18* allele) but not in strains having mutation in only one of these polymerases; this suggests that these two polymerases can compensate efficiently for each other. In contrast, the *in vitro* studies of Wang *et al.* (1993) suggest that DNA polymerase ε rather than δ is mainly responsible for the repair of lesions caused by UV and osmium tetroxide. Since no definitive role has been assigned to Pol ε in replication, it has been thought that the primary function of this enzyme is in replication-linked repair processes that are essential for cell viability (Stillman, 1994), and the results of Wang supported this idea well. However, *in vivo* studies provide no evidence for a role of Pol ε in repair. This enzyme is not involved in the repair of lesions caused by exogenous methylating agents (Blank *et al.*, 1994; Hałas *et al.*, 1997) or in post-replication repair of UV damaged DNA (Torres-Ramos *et al.*, 1997). In all these repair systems DNA polymerase δ is the main enzyme responsible for repair synthesis. Recent studies have also indicated the requirement of Pol δ in DNA mismatch repair and dispensability of Pol ε in this process (Longley *et al.*, 1997). Therefore, our observation that Pol ε is definitely required for the repair of lesions caused in DNA by UV irradiation seems to be important as giving an additional information about the role of Pol ε in DNA metabolism.

The third polymerase which was taken into consideration was the nonessential DNA polymerase ζ. This enzyme, after the conclusive work of Lawrence and his group (Morrison *et al.*, 1989; Nelson *et al.*, 1996) was generally accepted as the trans-lesion DNA polymerase, which explained perfectly its involvement in mutagenesis. However, we found that the deletion of the *REV3* gene prevents the repair of MMS-induced lesions and the double mutant strain carrying a mutation in the *POL3* gene and a deletion in the *REV3* gene arrests completely such repair. This result showed the direct involvement of DNA polymerase ζ in Methylated base Excision Repair system (MER). Since *rev3* mutants exhibit increased sensitivity not only toward MMS treatment but also toward UV-irradiation, we decided to include this polymerase in our investigations, although earlier results of Budd & Campbell (1995) gave us little hope for positive results. As expected, this polymerase does not participate in UV lesions repair. Since DNA polymerases α and β are not involved in repair of this lesions either, it should be accepted that only DNA polymerases responsible for chain elongation during replication are also responsible for filling gaps created in DNA during nucleotide excision repair. Therefore, specialisation of DNA polymerases in different mechanisms of DNA repair seems to be well consolidated.

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