Effects of DNA Double-Strand and Single-Strand Breaks on Intrachromosomal Recombination Events in Cell-Cycle-Arrested Yeast Cells

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ABSTRACT

Intrachromosomal recombination between repeated elements can result in deletion (DEL recombination) events. We investigated the inducibility of such intrachromosomal recombination events at different stages of the cell cycle and the nature of the primary DNA lesions capable of initiating these events. Two genetic systems were constructed in *Saccharomyces cerevisiae* that select for DEL recombination events between duplicated alleles of *CDC28* and *TUB2*. We determined effects of double-strand breaks (DSBs) and single-strand breaks (SSBs) between the duplicated alleles on DEL recombination when induced in dividing cells or cells arrested in G1 or G2. Site-specific DSBs and SSBs were produced by overexpression of the I-*Sce* I endonuclease and the gene II protein (gIIp), respectively. I-*Sce* I-induced DSBs caused an increase in DEL recombination frequencies in both dividing and cell-cycle-arrested cells, indicating that G1- and G2-arrested cells are capable of completing DSB repair. In contrast, gIIp-induced SSBs caused an increase in DEL recombination frequency only in dividing cells. To further examine these phenomena we used both γ-irradiation, inducing DSBs as its most relevant lesion, and UV, inducing other forms of DNA damage. UV irradiation did not increase DEL recombination frequencies in G1 or G2, whereas γ-rays increased DEL recombination frequencies in both phases. Both forms of radiation, however, induced DEL recombination in dividing cells. The results suggest that DSBs but not SSBs induce DEL recombination, probably via the single-strand annealing pathway. Further, DSBs in dividing cells may result from the replication of a UV or SSB-damaged template. Alternatively, UV induced events may occur by replication slippage after DNA polymerase pausing in front of the damage.

EUKARYOTIC genomes contain both unique DNA sequences as well as repeated ones. Repeated sequences may occur on different DNA molecules or on the same molecule and may be either clustered or scattered. Recombination between such repeated sequences on the same DNA molecule can generate genome rearrangements such as deletions or gene amplifications (Petes and Hill 1988; Klein 1995). Such processes are important in evolution (Guttman and Dykhuizen 1994; Shapiro 1992) carcinogenesis, *e.g.*, (Tlsty *et al.* 1995), aging and genetic diseases. Hence it is important to determine the mechanism and specific DNA lesions that lead to the increase of such intrachromosomal recombination events. The yeast *Saccharomyces cerevisiae* provides an excellent model system for such studies. Intrachromosomal- or plasmid-based recombination events resulting in deletions have been studied with several constructs, *e.g.*, Fasullo and Davis 1987; Rudin and Haber 1988; Schiestl *et al.* 1988; Aguilera and Klein 1989; Nickoloff *et al.* 1989; Fishman-Lobell *et al.* 1992). Recombination events depend on homologous DNA substrate length as indicated by two studies which show that decreasing homology length from about 1000 bp to about 250 bp reduces the frequency of deletions (Yuan and Keil 1990; Jinks-Robertson *et al.* 1993). Another study found that as little as 63 to 89 bp of homology were sufficient for DSB-induced recombination between repeats and that the frequency was linearly dependent on homology length (Sugawara and Haber 1992).

Several mechanisms have been proposed to account for intrachromosomal recombination events between direct repeats that generate deletions (Schiestl *et al.* 1988; Haber 1992; Lovett *et al.* 1993; Belmaaza and Chartrand 1994; Klein 1995; Galli and Schiestl 1995a). Intrachromosomal recombination events may occur by recombination between the two repeats within one chromatid via intrachromatid exchange, single-strand annealing, one-sided invasion events or replication slippage. Such events may also occur by recombination between sister chromatids via unequal sister-chromatid exchange or sister-chromatid conversion. Intrachromatid exchange and sister-chromatid exchange events are termed conservative events when both reciprocal products can be recovered, whereas in nonconservative events only one of the two products can be recovered. Conservative events may involve strand exchange and

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Holliday junction resolution steps proposed in the Meselson-Radding (Meselson and Radding 1975) or the double-strand break (DSB) repair models (Szostak *et al.* 1983).

Intrachromatid exchange occurs by reciprocal crossing-over between the direct repeats leaving a single copy of the gene on the chromosome while an excised circular fragment bears the second copy of the gene ("pop-out events"). Originally, it was thought that this form of reversion would account for most of these deletion recombination events. Schiestl *et al.* (1988) investigated the contribution of this mechanism to the frequency of such intrachromosomal recombination events by placing an origin of replication within the duplicated region such that excised plasmid products could be recovered. From these experiments only a minority of events (about 1%) can be explained by the "pop-out" mechanism, suggesting that the majority of these events result from a nonconservative mechanism. With a different system that forced amplification of the excised circle Santos-Rosa and Aguilera (1994) found that fewer than 10% of the deletion events produced circles reinforcing the previous report. Such a nonconservative mechanism for deletion recombination was supported by Fishman-Lobell *et al.* (1992) when they failed to find reciprocal recombination products by physical analysis of recombination intermediates.

Models proposed for intrachromosomal recombination include a nonconservative pathway termed single-strand annealing in mammalian cells (Lin *et al.* 1984; Lin *et al.* 1990) and in yeast (reviewed by Haber 1992). Single-strand annealing is initiated by a double-strand break (DSB) in a nonhomologous region between repeats or within one repeat (Fishman-Lobell *et al.* 1992). DNA degradation of single strands from exposed 5′ ends of DSBs leads to single-stranded regions which anneal with each other once the degradation has exposed the repeated sequences. The 3′ tails are processed and nicks are ligated thus producing the deletion. Another nonconservative recombination mechanism, one-sided invasion, involves an initiating DSB within one of the duplicated homologous sequences. Five prime to 3′ degradation of the exposed end would be followed by invasion of the 3′ single strand in the homologous region. The resultant D-loop formation could be resolved by continuation of the 5′ degradation, single-strand nick formation and DNA repair synthesis. Nonconservative recombination events can also be produced by replication slippage of one polymerase or by pairing of the two replicating sister strands at a stalled replication fork (Lovett *et al.* 1993).

Intrachromosomal deletions can also be products of unequal sister-chromatid exchange or sister-chromatid conversion events. Unequal sister-chromatid exchanges (SCEs) give rise to a duplication of any sequence between the repeated sequence (Schiestl *et al.* 1988; Galli and Schiestl 1995a). The portion of SCE events was determined by assaying for reciprocal products (Schiestl *et al.* 1988). If an SCE event occurs at the two- to eight-cell stage of a colony, a cell with only one copy of the repeat and another cell with the reciprocal triplication of the repeat should be produced. Both of these cells should subsequently produce equally sized sectors in the growing colony. In fact, only about 4% of the recombination events were observed to produce such a reciprocal triplication. In another study that specifically selected for SCEs at several loci including the *HIS3* locus (Fasullo and Davis 1987) it was demonstrated that such events occurred at a frequency of 4×10^{-6}. In comparison, a deletion specific recombination substrate at the *HIS3* locus exhibits deletion recombination events at a frequency of 3×10^{-4} (Schiestl *et al.* 1988). These results suggest that the majority of deletion recombination events are not due to unequal sister-chromatid exchanges.

The DNA recombination frequency can be enhanced by DNA damage (Zimmermann 1973; Schiestl 1989; Hoffmann 1994; Friedberg *et al.* 1995) or by so-called natural recombinators (Strathern *et al.* 1991). Radiation, such as ultraviolet light (UV) and X-rays have been extensively used to induce recombination events. UV irradiation causes various photoproducts, the repair of which introduces single-strand breaks. Ionizing radiation also causes a variety of base damages and SSBs but the biologically most significant lesions are double-strand breaks (Friedberg *et al.* 1995).

Natural recombinators are categorized into several groups by their mechanism. One group consists of site-specific recombinases such as FLP recombinase which catalyzes 2-μm circle plasmid inversion (Futcher 1988), the lambda int family (Landy 1993) and P1 recombinase cre which recognizes lox sites (Sauer 1987). Another group of natural recombinators is composed of enzymes catalyzing site-specific DSBs and SSBs at specific DNA sequences. Enzymes, such as HO and I-*Sce* I, catalyzing site-specific DSBs induce recombination between homologs (Nickoloff *et al.* 1986; Fairhead and Dujon 1993) and between Ty elements (Parket *et al.* 1995). Such DSBs are powerful inducers of deletion events between repeated elements (Rudin and Haber 1988; Nickoloff *et al.* 1989; Fishman-Lobell *et al.* 1992; Plessis *et al.* 1992). Single-strand breaks like those induced by the gene II protein (gIIp) can also lead to interchromosomal recombination events (Strathern *et al.* 1991).

Several groups have investigated the cell-cycle dependence of induced recombination. Unequal sister-chromatid recombination events are limited to the S or the G2 phase (Fasullo and Davis 1987; Jackson and Fink 1981; Kadyk and Hartwell 1992) while recombination between homologs may occur in G1 (Esposito 1968; Fabre 1978). X-rays induce much higher levels of interchromosomal recombination in G1 than G2 (Esposito 1968; Fabre *et al.* 1984). This can be explained by the

preferential use of sister chromatids over homologs as recombination substrates in G2 (Fabre *et al.* 1984; Kadyk and Hartwell 1992).

The system to study deletion formation by intrachromosomal recombination reported by Schiestl *et al.* (1988) was constructed by integration of plasmid pRS6, containing the *LEU2* gene and an internal fragment of *HIS3*, into the genomic *HIS3* gene. This resulted in a disruption of the *HIS3* gene with duplicate *HIS3* sequences flanking the disruption, *i.e.*, *his3Δ3′-LEU2*-pBR322-*his3Δ5′*. The two portions of *his3* share about 400 bp of homology and recombine with each other to produce His^+ revertants at a spontaneous frequency of about 1×10^{-4} (Schiestl *et al.* 1988). Ninety-nine percent of all reversion events involve loss of the integrated plasmid. These intrachromosomal recombination events are termed DEL recombination events (Schiestl 1989) to differentiate these events from intrachromosomal gene conversion events that do not involve deletions, *e.g.*, Jackson and Fink 1981.

We were interested in discovering what type of DNA damage can induce DEL recombination at which cell-cycle stage. To do that we constructed isogenic strains with temperature-sensitive alleles of genes required for cell-cycle progression and recombination substrates of these same genes on the homologs. Cells arrested at the restrictive temperature, therefore, have to undergo a reversion event, by recombination or mutation, to overcome the otherwise terminal cell-cycle block and develop into colonies. We determined the effects of UV, γ-rays, a site-specific DNA DSB or a site-specific DNA single-strand break (SSB) on intrachromosomal DEL recombination events in cells arrested in G1 or G2 versus dividing cells.

MATERIALS AND METHODS

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

Plasmids: *Construction of plasmids pAG2AS and pAG3 containing an I-Sce I site and M13ori, respectively, for insertional duplication-disruption of the CDC28 gene:* Plasmid pAG2 was constructed by inserting into the *Eco*RI site of YIplac211 (Gietz and Sugino 1988) a 616-bp *Msc*I fragment of the *CDC28* ORF from plasmid YRp7 CDC28-4 which was kindly provided by S. Reed (Reed *et al.* 1982). The 499-bp *Eco*RI-*Ssp*I fragment of the *CDC28* ORF from pAG2 was then subcloned into the *Eco*RI-*Sma*I sites of YIplac211 yielding plasmid pAG2A. Via a linker the *Ssp*I site of pAG2A was converted to a *Bgl*II site which was then used for the insertion of the I-*Sce* I megalinker (Boehringer-Mannheim, Indianapolis) to form plasmid pAG2AS.

Plasmid pAG2A was digested with *Ahd*I-*Hin*dIII, and the 3-kb fragment containing the *URA3* gene and 499-bp of CDC28 was isolated. A 1600-bp *Ahd*I-*Hin*dIII fragment from Litmus 28 (New England Biolabs, Beverly, MA) containing the M13ori was isolated and ligated with the 3-kb *Ahd*I-*Hin*dIII fragment of pAG2A to yield pAG3.

Construction of plasmids pAGT and pAG4 containing the I-SceI site and the M13ori, respectively, for insertional duplication-disruption of the TUB2 gene: Plasmid pRB121 containing 532-bp of the *TUB2* ORF was obtained from D. Botstein (Neff *et al.* 1983) and its *Nru*I site was converted to a *Sal*I site. The 925-bp *Eco*RI-*Sal*I fragment of pRB121, containing 532 bp of *TUB2* ORF, was ligated with the 3.9-kb *Eco*RI and *Sal*I fragment of plasmid pAG2AS, containing the plasmid backbone with the *URA3* gene and the I-*Sce* I site, to produce plasmid pAGT that contains the internal fragment of *TUB2*, *URA3* and the I-*Sce* I site. The same 925-bp *Eco*RI-*Sal*I fragment was also ligated into the 4.5-kb *Eco*RI-*Xho*I plasmid backbone of pAG3 containing the *URA3* gene and the M13ori to yield pAG4.

Construction of pAG7 for galactose-inducible expression of geneII protein (gIIp): pMA32 (obtained from Manivasakam Palaniyandi) contains the *Eco*RI-*Bam*HI sequence of the yeast *GAL1,10* promoter (Johnston and Davis 1984) cloned into the multicloning site of pUC19. The gIIp DNA sequence was cloned as a 2-kb *Bam*HI-*Pst*I fragment from pDG117 IIA (kindly provided by P. Model) into corresponding sites in pMA32 to form plasmid pAGgal. The 1.9-kb *Eco*RI fragment of pAGgal containing the gIIp under the *GAL1* promoter was cloned into YCplac111 (*CEN4*, *LEU2*; Gietz and Sugino 1988) to produce plasmid pAG7.

Strains: The genotypes of strains of *S. cerevisiae* are listed in Table 1. The haploid strains AGY2 (*tub2-104*; Galli and Schiestl 1995b) and AGY7 (*cdc28-4*; Galli and Schiestl 1996) are isogenic and were constructed previously by two-step gene replacement. Crosses of these strains with strain RSY6 gave strains AGY4 and AGY8, respectively. Further modification (see below) of these strains yielded strains AGY5 and AGY9, respectively, which are isogenic to each other and to RS112 (Schiestl *et al.* 1988; Schiestl 1989). Strain YWY200, constructed by Wendy Yap (W. Yap and R. H. Schiestl, unpublished results), has integrated into the *LYS2* locus plasmid pWY203 containing the I-*Sce* I gene under the *GAL1* promoter. It was then crossed with AGY2 and AGY7 to yield AGY11 and AGY12, respectively, which are thus isogenic to each other. All other diploid strains used (AGY13 through AGY28) are isogenic to AGY11 and AGY12.

The diploid strains AGY9, AGY15, AGY16, AGY24, AGY24A, AGY26, AGY26A, and AGY28 are temperature-sensitive because the *cdc28-4* mutation allows G1 arrest at 37° (Reed 1980). Strains AGY5, AGY13, AGY14, AGY25, AGY25A, AGY27, and AGY27A are cold sensitive due to the *tub2-104* mutation that allows G2 arrest at 12° (Thomas *et al.* 1985).

Strains AGY8, AGY12, and AGY22 were transformed with pAG2A linearized by *Sac*I. Ura^+ transformants were selected and screened for *cdc28-4* mutations via cell-cycle arrest at the restrictive temperatures. The resulting strains, AGY9, AGY15, and AGY24A, have on one chromosome the *cdc28-4* allele and on the homolog the duplication-disruption alleles of *cdc28* (Figure 1A).

Likewise strains AGY4, AGY11, and AGY23 were transformed with pRB121 linearized by *Bam*HI. Ura^+ transformants were selected and screened for *tub2-104*. Hence AGY5, AGY14, and AGY25A contain on one chromosome the *tub2-104* allele and on the homolog the duplication-disruption alleles of *tub2* (Figure 1B).

Strains AGY13 and AGY16 contain the I-*Sce* I recognition site between the duplication-disruption alleles of *TUB2* and *CDC28*, respectively, via transformations into AGY11 and AGY12 of plasmids pAGT and pAG2AS and were screened as described above.

Strains AGY24 and AGY25, which contain the origin of

TABLE 1

List of *Saccharomyces cerevisiae* strains

Name	Cross or parent strain	Genotype or construction	Source or reference
RSY6		*MAT***a** *ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 HIS3::pRS6*	Schiestl *et al.* (1988)
RSY12		*MAT***a** *leu2-3,112 his3-11,15 URA3::HIS3*	Schiestl and Petes (1991)
AGY2		*MAT*α *ura3-52 leu2*Δ*98 ade2-101 lys2-801 his3*Δ*200 tub2-104*	Galli and Schiestl (1995b)
AGY4	RSY6 × AGY2	*MAT***a**/*MAT*α *ura3-52/ura3-52 leu2-3,112/leu2*Δ*98 trp5-27/TRP5 ade2-40/ade2-101 ilv1-92/ILV1 lys2-801/LYS2 arg4-3/ARG4 his3*Δ *200/HIS3*::pRS6 *tub2-104/TUB2*	This study
AGY7		*MAT*α *ura3-52 leu2*Δ*98 ade2-101 lys2-801 his3*Δ*200 cdc28-4*	Galli and Schiestl (1996)
YWY200	RSY12	With *LYS2*::pWY203 (*GAL1-I-Sce I*)	Wendy Yap
AGY5	AGY4	With *TUB2*::pRB121	This study
AGY8	RSY6 × AGY7	*MAT***a**/*MAT*α *ura3-52/ura3-52 leu2-3,112/leu2*Δ*98 trp5-27/TRP5 ade2-40/ade2-101 ilv1-92/ILV1 arg4-3/ARG4 lys2-801/LYS2 his3*Δ*200/HIS3*::pRS6 *cdc28-4/CDC28*	This study
AGY9	AGY8	With *CDC28*::pAG2A	This study
AGY11	YWY200 × AGY2	*MAT***a**/*MAT*α *leu2-3,112/leu2*Δ*98 his3-11,15/his3*Δ*200 ura3-52/ URA3::HIS3 LYS2*::pWY203/*lys2-801 ade2-101/ADE2 tub2-104/ TUB2*	This study
AGY12	YWY200 × AGY7	*MAT***a**/*MAT*α *leu2-3,112/leu2*Δ*98 his3-11,15/his3*Δ*200 ura3-52/ URA3::HIS3 LYS2*::pWY203/*lys2-801 ade2-101/ADE2 cdc28-4/ CDC28*	This study
AGY13	AGY11	With *TUB2*::pAGT	This study
AGY14	AGY11	With *TUB2*::pRB121	This study
AGY15	AGY12	With *CDC28*::pAG2A	This study
AGY16	AGY12	With *CDC28*::pAG2AS	This study
AGY22	RSY12 × AGY7	*MAT***a**/*MAT*α *leu2-3,112/leu2*Δ*98 his3-11,15/his3*Δ*200 ura3-52/ URA3::HIS3 ade2-101/ADE2 lys2-801/LYS2 cdc28-4/CDC28*	This study
AGY23	RSY12 × AGY2	*MAT***a**/*MAT*α *leu2-3,112/leu2*Δ*98 his3-11,15/his3*Δ*200 ura3-52/ URA3::HIS3 ade2-101/ADE2 lys2-801/LYS2 tub2-104/TUB2*	This study
AGY24	AGY22	With *CDC28*::pAG3	This study
AGY24A	AGY22	With *CDC28*::pAG2A	This study
AGY25	AGY23	With *TUB2*::pAG4	This study
AGY25A	AGY23	With *TUB2*::pRB121	This study
AGY26	AGY22	With *CDC28*::pAG3, pAG7	This study
AGY26A	AGY22	With *CDC28*::pAG3, YCplac111	This study
AGY27	AGY23	With *TUB2*::pAG4, pAG7	This study
AGY27A	AGY23	With *TUB2*::pAG4, YCplac111	This study
AGY28	AGY22	With *CDC28*::pAG2A, with pAG7	This study

replication of the M13 phage, were constructed by insertion of the plasmid pAG3 and pAG4 followed by the screening for the temperature-sensitive phenotype conferred by the *cdc28-4* or *tub2-104* alleles, respectively.

Strains AGY26, AGY27, and AGY28 were obtained by transforming AGY24, AGY25, and AGY24A with the centromeric plasmid pAG7 which contains the gIIp sequence under the *GAL1,10* promoter and the *LEU2* marker.

Strains AGY26A and AGY27A were constructed by transforming AGY24 and AGY25 with YCp*lac*111 (*CEN, LEU2*).

Cell cycle arrest: The cells of the yeast *S. cerevisiae* grow by budding and the bud emergence is a landmark of the initiation of DNA synthesis (Pringle and Hartwell 1981). Cells in G1 are unbudded and cells in G2 have buds of the same size as the mother cell but with only one nucleus. The cell cycle synchronization of yeast cultures was checked by microscopic observation.

Cells of strain AGY9 were synchronized in G1 by incubation in YPAD at 37° for 2 hr under constant shaking. Cells of other strains carrying the *cdc28-4* mutation were arrested in G1 by incubation at 37° for 3–4 hr in SC-ura; cells carrying plasmids pAG7 or YCp*lac*111 were incubated in SC-ura-leu. The *cdc28-4* mutants arrest as large unbudded cells (Reed 1980). The percentage of the arrested cells was determined after counting at least 200 cells. Experiments were performed only with cultures in which more than 95% of the cells showed the respective G1 phenotype.

Cells of AGY5 were arrested in G2 by incubation of log phase cells at 12° in YPAD for 32 hr. All other cells carrying the *tub2-104* mutation were arrested in G2 by incubation at 12° in SC-ura, while cells carrying plasmids pAG7 or YCp*lac*111 were incubated in SC-ura-leu. G2 arrest was checked by counting at least 200 cells per culture, and experiments were performed only with cultures in which more than 95% of the cells showed the respective G2 phenotype.

Determination of intrachromosomal (DEL) recombination events: DEL recombination events in G1 were determined as follows: Single colonies of strain AGY9 were inoculated into

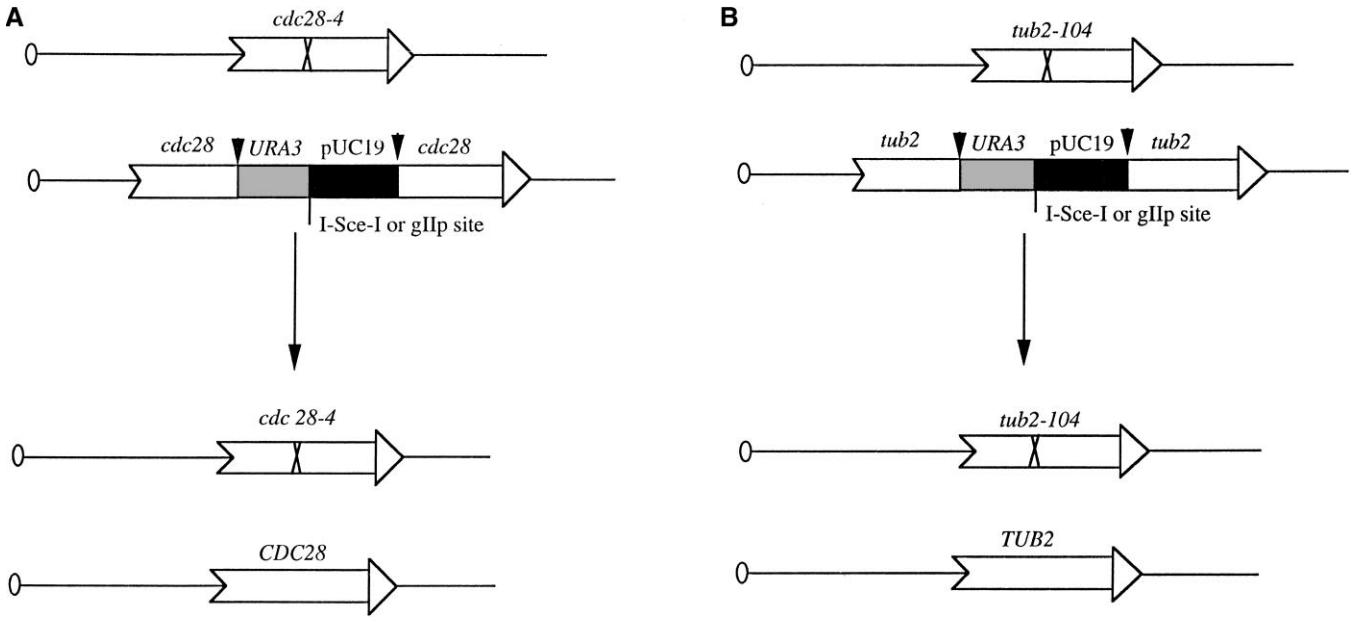

Figure 1.—(A) System used to select for intrachromosomal DEL recombination in G1. Strain AGY9 contained the *cdc28* duplication-disruption on chromosome *II* and the *cdc28-4* mutation on the homolog. (B) System used to select for intrachromosomal DEL recombination in G2. AGY5 contained the *tub2* duplication-disruption on chromosome *VI* and the *tub2-104* mutation on the homolog. In both cases DEL recombination events give rise to temperature resistant Ura$^-$ colonies.

YPAD and grown at 25° for 24–48 hr. Five-milliliter aliquots containing 2–3 $\times$ 10^7 cells/ml were arrested in G1 as described above. The G1 arrested cultures were irradiated at 37° with UV or γ-rays as previously reported (Galli and Schiestl 1995a). UV-irradiation was carried out in liquid medium in Petri dishes under constant shaking in the dark, using a UV irradiator at the dose rate of 3.5 ergs/m^2/sec. Cells were exposed to γ-rays using a ^{60}Co γ-ray source at a dose rate of 12.2 cGy per second. After irradiation, cells were washed in 37° sterile distilled water and counted, and appropriate numbers were plated onto prewarmed YPAD plates. The plates were incubated at 37° for 2–3 days and the number of Cdc28$^+$ colonies, which included intrachromosomal recombinants, interchromosomal recombinants, and revertants of *cdc28-4*, was determined. Among those *Cdc28* colonies, the frequency of intrachromosomal recombinants was determined as the fraction of uracil-requiring colonies (Figure 1) by replica plating onto SC-ura plates. To determine viability, an aliquot from a different dilution of the same culture was plated onto YPAD medium and incubated at 25° for 4 days.

To determine DEL recombination events in G2, single AGY5 colonies were inoculated into YPAD and grown at 30° for 17–24 hr. Five-milliliter aliquots containing 2–3 $\times$ 10^7 cells/ml were arrested in G2 as described above and then irradiated at 12° with UV or γ-rays as described before. Cells were washed in 12° sterile distilled water, counted and plated onto precooled YPAD plates. The plates were incubated at 12° for 16–20 days, and the number of *TUB2* colonies determined as described above for G1-arrested cells. To determine viability, an aliquot from a different dilution of the same culture after irradiation was plated onto YPAD medium and incubated at 30° for 3 days.

For determining the frequency of DEL events in dividing cells single colonies of AGY5 and AGY9 were inoculated into YPAD and grown to a concentration of 2 $\times$ 10^7 cells/ml. Five-milliliter aliquots (2–3 $\times$ 10^7 cells/ml) were exposed to UV and γ-rays, washed, counted and plated at the respective restrictive temperature as described above. For AGY9, DEL recombinants were counted as *CDC28 ura3* colonies and for AGY5, as *TUB2 ura3* colonies.

Induction of a site-specific DNA-double strand break: Strains AGY13, AGY14, AGY15 and AGY16 have the gene encoding the I-*Sce* I endonuclease under the yeast *GAL1* promoter integrated at the *LYS2* locus. Single colonies of these strains grown on YPAD plates were inoculated into SC-ura 2% glucose (about 2 $\times$ 10^5 cells/ml) and grown for 17 hr with constant shaking at the permissive temperature. Single colonies of these strains were also inoculated into SC-ura 5% galactose either at 30° for 24 hr (AGY13 and AGY14) or at 25° for 32 hr (AGY15 and AGY16). During this time cells underwent 4–5 generations. Thereafter, cells were washed, counted, and DEL recombination frequencies determined as described above.

To determine the effects of I-*Sce* I expression in G1-arrested cells 10 ml aliquots of cultures containing 2–3 $\times$ 10^7 cells/ml of AGY15 and AGY16 were arrested in G1 in SC-ura 2% raffinose for 4 hr at 37°. Each culture was then split into two aliquots of 5 ml each. The two cultures were washed with warm distilled water and one of the cultures was exposed to SC-ura 2% glucose and the other culture to SC-ura 5% galactose. At different time points aliquots were washed and DEL recombination frequencies were determined as described above.

To determine effects of I-*Sce* I expression in G2 arrested cells, 10 ml aliquots of culture containing 2–3 $\times$ 10^7 cells/ml of AGY13 and AGY14 were arrested in G2 in SC-ura 2% raffinose for 36 hr at 12°. Then, each culture was split into two aliquots of 5 ml each. The two cultures were washed with cold distilled water and one of the cultures was exposed to SC-ura 2% glucose and the other culture to SC-ura 5% galactose. At different time points aliquots were washed and DEL recombination frequencies were determined as described above.

Induction of a site-specific DNA-single strand break: AGY26, AGY27 and AGY28 carry the centromeric plasmid pAG7. This plasmid contains the gIIp DNA sequence under the yeast *GAL1* promoter and the *LEU2* marker for selection. AGY26A

and AGY27A contain the centromeric vector YCplac111 without the gIIp sequence as control.

In the experiments with dividing cells, 2×10^5 cells/ml of AGY27 and AGY27A strains were inoculated into SC-ura-leu 2% glucose or SC-ura-leu 5% galactose and grown at 30° for 24 hr under constant shaking. For the strains AGY26, AGY26A and AGY28, cells were grown at 25° for 36 hr. During this time cells underwent four to five generations. Thereafter, cells were washed, counted and DEL recombination frequencies were determined as described above.

In the experiments with G1-arrested cells, single colonies of AGY26, AGY26A, and AGY28 were grown in SC-ura-leu 2% raffinose for 48 hr at 25°. Fifteen-milliliter aliquots of culture containing 2–3 $\times$ 10^7 cells/ml were arrested in G1 in fresh SC-ura-leu medium containing 2% raffinose for 4 hr at 37°. Each culture was split into three aliquots of 5 ml each. To the first culture galactose was added to 2% and to the second culture glucose to 2%. The third culture was kept in raffinose. At different time points aliquots were washed and DEL recombination in G1 was determined as described above.

For experiments with G2 arrested cells, single colonies of AGY27 and AGY27A were grown in SC-ura-leu 2% raffinose for 24–32 hr at 30°. Fifteen-milliliter aliquots of culture containing 2–3 $\times$ 10^7 cells/ml were arrested in G2 in fresh SC-ura-leu medium containing 2% raffinose for 48 hr at 12°. Each culture was split into three aliquots of 5 ml each. To the first culture galactose was added to 2% and to the second culture glucose to 2%. The third culture was kept in raffinose. At different time points, aliquots were washed and DEL recombination in G2 was determined as described above.

RESULTS

Intrachromosomal (DEL) recombination in G1 and in G2: In the present study we linked the means to arrest cells in a specific cell-cycle phase with a marker for recombination selection to assure that the induced recombination events actually happened in that particular cell-cycle phase. To do this, we used genes required for progression of cells through the cell cycle to construct isogenic diploid strains with temperature-sensitive alleles and recombination substrates of the same genes on the homologs. Therefore, cells arrested and kept at the restrictive temperature have to undergo reversion by recombination or mutation to overcome the otherwise terminal cell-cycle block and to develop into colonies.

Strain AGY9 was designed to determine the frequency of DEL recombination events in G1. This diploid strain was constructed by inserting a plasmid containing an internal fragment of *CDC28* resulting in a duplication-disruption of the *CDC28* gene which was used as the DEL recombination substrate. The other homolog contained the *cdc28-4* mutation that allowed G1 arrest at the restrictive temperature of 37°. The *URA3* marker on the integrated plasmid is flanked by the *CDC28* duplication allele (*cdc28*Δ*3′-URA3*-pUC19-*cdc28*Δ*5′*). The *cdc28* alleles share 499 bp of homology, and recombination between them yields *CDC28* as well as loss of *URA3* and resistance to 5-fluoroorotic acid (5-FOA, Figure 1A).

Strain AGY5 was constructed to determine the frequency of DEL recombination events in G2. This diploid strain was constructed by disrupting one copy of the *TUB2* sequence by inserting a plasmid containing an internal fragment of *TUB2* resulting in a duplication-disruption gene, which was used as the DEL recombination substrate. The other homolog contained the cold-sensitive *tub2-104* allele that allowed G2 arrest at the restrictive temperature of 12°. The *URA3* marker on the integrated plasmid was flanked by the *TUB2* duplication alleles resulting in the construct *tub2*Δ*3′-URA3*-pUC19-*tub2*Δ*5′*. The *tub2* alleles share 532 bp of homology and recombination between them yields *TUB2* as well as loss of *URA3* and resistance to 5-FOA (Figure 1B).

Cells of strains AGY9 and AGY5 were arrested at the restrictive temperature in G1 or G2. Microscopic examination revealed that after cell-cycle arrest 96.6 $\pm$ 0.8% of cells of strain AGY9 were in G1 and 98.4 $\pm$ 0.4% of cells of strain AGY5 were in G2. About 4×10^{-5} spontaneous reversions to $Cdc28^+$ or $Tub2^+$ appeared. These colonies may include the following: (1) *intra*chromosomal recombinants that have deleted the disrupting fragment and that reconstitute the *CDC28* or *TUB2* gene (Figure 1); (2) reverse mutations of the temperature-sensitive mutant alleles on the homologs; and (3) *inter*-chromosomal recombinants. Intrachromosomal DEL recombinants should always have lost the *URA3* marker between the two copies of the gene duplication, whereas reverse mutation events should have maintained the *URA3* marker. Most interchromosomal recombination events should also maintain the *URA3* marker; however, it is possible for interchromosomal gene conversion events to loose the marker. Interchromosomal gene conversion events occur spontaneously at a frequency of about 1 to 10×10^{-6} (Fabre 1978; Schiestl 1989), whereas DEL recombination events are about 10-fold more frequent. In the tables we report only the frequency of $Cdc28^+$ $Ura3^-$ or $Tub2^+$ $Ura3^-$ colonies, which should mainly occur by intrachromosomal DEL recombination.

Effect of a site-specific DNA DSB on DEL recombination frequencies: A system based on the overexpression of the rare cutting endonuclease I-*Sce* I was used to study induction of DEL recombination by a single site-specific DSB. Yeast mitochondria contain the I-*Sce* I endonuclease, which is responsible for intron mobility (Dujon 1989). The I-*Sce* I recognition cutting site is an 18-bp DNA sequence which is not present in the yeast nuclear genome (Plessis *et al.* 1992). Several studies reported that I-*Sce* I cuts when its recognition site is present either in nuclear or episomal yeast DNA (Fairhead and Dujon 1993; Plessis *et al.* 1992). Strains AGY13, AGY14, AGY15 and AGY16 contain the I-*Sce* I endonuclease coding sequence integrated in the nuclear genome at the *LYS2* locus and regulated by the *GAL1,10* promoter. AGY13 and AGY16 contain the I-*Sce* I recognition site in between two copies of the *TUB2* and *CDC28* duplication-disruption alleles, respectively. In both cases, the I-*Sce* I site was placed at equivalent positions between pUC19 and *URA3*. We first determined DEL recombination

TABLE 2

Effect of I-*Sce* I and gene II protein expression on DEL recombination in dividing cells

Strain	Rel. genotype	DEL events/10^4 survivors		
		Glucose	Galactose	Fold increase Gal/Glu
AGY14	*tub2-104/tub2*Δ-*URA3-tub2*Δ	0.96 ± 0.18	1.28 ± 0.09	1.3
AGY13	*tub2-104/tub2*Δ-*URA3*-I *Sce* I-*tub2*Δ	1.34 ± 0.86	4143 ± 716	3092**
AGY15	*cdc28-4/cdc28*Δ-*URA3-cdc28*Δ	0.98 ± 0.16	1.08 ± 0.25	1.1
AGY16	*cdc28-4/cdc28*Δ-*URA3*-I *Sce* I-*cdc28*Δ	5.1 ± 2.45	7296 ± 1035	1431**
AGY26A	*cdc28-4/cdc28*Δ-*URA3*-M13ori-*cdc28*Δ, YCplac111	0.56 ± 0.10	0.65 ± 0.22	1.2
AGY26	*cdc28-4/cdc28*Δ-*URA3*-M13ori-*cdc28*Δ, pAG7	1.67 ± 0.66	22.5 ± 5.3	13.5*
AGY27A	*tub2-104/tub2*Δ-*URA3*-M13ori-*tub2*Δ, YCplac111	1.08 ± 0.56	1.00 ± 0.25	0.9
AGY27	*tub2-104/tub2*Δ-*URA3*-M13ori-*tub2*Δ, pAG7	0.42 ± 0.18	1.36 ± 0.12	3.2*
AGY28	*cdc28-4/cdc28*Δ-*URA3-cdc28*Δ, pAG7	0.77 ± 0.13	0.86 ± 0.09	1.1

1×10^7 cells/ml were inoculated in 5 ml SC-URA 2% glucose and SC-URA 5% galactose and incubated at 30° temperature for 24–32 hr while shaking. Then, cells were washed, counted, and plated. Data are reported as the mean of three independent experiments ± the standard error. Results were statistically analyzed using Student's *t*-test.

*$P < 0.005$, **$P < 0.001$.

frequencies in dividing cells grown in glucose versus galactose (Table 2). Growth of strains AGY16 (containing the *CDC28* duplication) and AGY13 (containing the *TUB2* duplication) on SC-ura 5% galactose resulted in increases of DEL recombination frequencies of 3000- and 1400-fold in comparison to growth on glucose (Table 2). When isogenic control strains AGY14 and AGY15, which contained the I-*Sce* I gene under the *GAL1* promoter but not the I-*Sce* I recognition site, were grown on galactose medium there were no increases in DEL recombination frequencies (Table 2). To study the effects of a double-strand break on recombination in cell-cycle-arrested cells, we determined DEL recombination frequencies in cells arrested in G1 or G2 phase and thereafter exposed to glucose or galactose under restrictive growth conditions (Figure 2). Cells from AGY16 arrested in G1 and from AGY13 arrested in G2 and then exposed to 5% galactose under cell-cycle arrest resulted in a time-dependent increase in DEL recombination frequency (Figure 2). AGY14 and AGY15 strains contain the I-*Sce* I gene under the *GAL1* promoter but lack the I-*Sce* I recognition site. Exposure of these cell-cycle-arrested cells to galactose did not result in any induction of DEL recombination (data not shown). These results indicate that a site-specific DNA DSB is able to induce DEL recombination in dividing as well

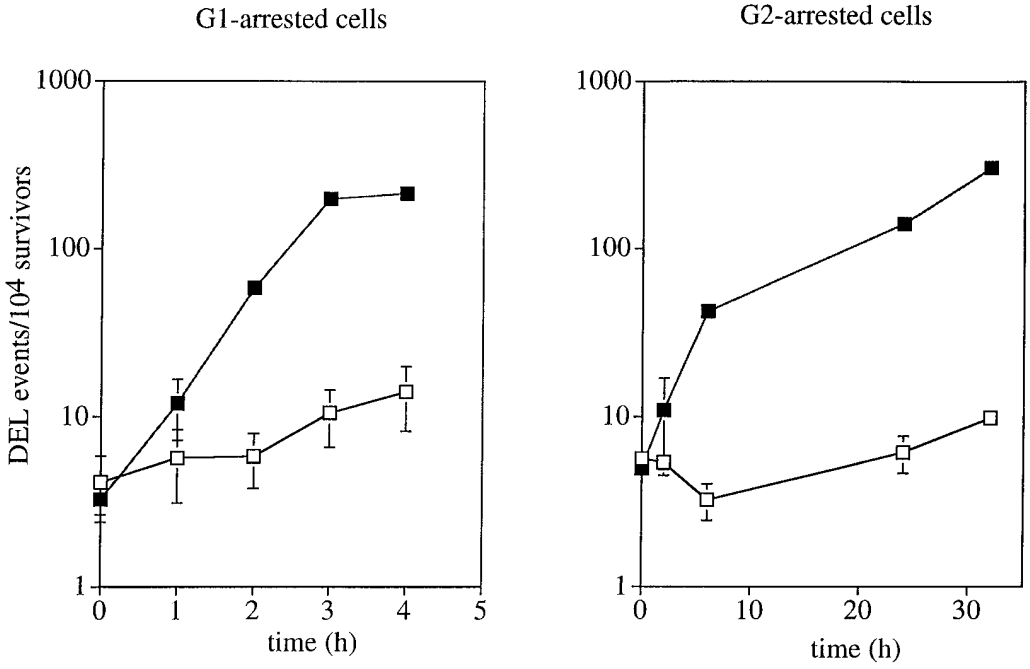

Figure 2.—Effect of I-*Sce* I overexpression on DEL recombination in G1- and G2-arrested cells. Cells of AGY13 and AGY16 strains were cell-cycle arrested, as described in the materials and methods, and exposed to 2% glucose (open squares), or 5% galactose (solid squares) at time 0. At different time points DEL recombination was measured. Results are reported as the mean of three independent experiments ± standard error.

as in G1 and G2 cell-cycle-arrested cells, or in other words, G1- as well as G2-arrested cells are capable of completing DSB repair.

Effect of a site-specific DNA SSB on DEL recombination frequencies: The gene II protein (gIIp) of the filamentous coliphages (f1, fd and M13) is a multifunctional protein required for DNA replication. It initiates DNA synthesis by producing a SSB in the origin of replication (Fulford and Model 1988). The M13 origin of replication contains the 37-bp sequence which represents the minimal recognition site for nicking (Cleary and Ray 1981; Dotto *et al.* 1984; Zinder and Horiuchi 1985). Expression of gIIp in a diploid yeast strain containing the gIIp recognition site in a genetically marked interval increases gene conversion and crossing-over within the same interval (Strathern *et al.* 1991). Strains AGY26, containing the *CDC28* duplication, and AGY27, containing the *TUB2* duplication, were constructed to determine DEL recombination frequencies induced by site-specific SSBs. These strains contained the M13 origin of replication between the gene-duplication alleles, as well as a centromeric plasmid (pAG7) containing the *GAL1*-inducible gIIp construct. Within the DEL recombination constructs the gIIp sites were placed between pUC19 and *URA3*, the same position as the I-*Sce* I site in the above experiment. The site is oriented so that the gIIp nicks are made in the bottom strands in the constructs shown in Figure 1. Comparison of the growth of these strains in galactose versus glucose resulted in a 13-fold increase in DEL recombination for AGY26 and 3-fold for AGY27 (Table 2). Growth of control strains AGY26A and AGY27A (containing the gIIp site in the recombination substrates and the vector YCp*lac*111 without the *GAL1-gII*p insert) in galactose did not result in any increase in DEL recombination frequency (Table 2). Growth in galactose of another control strain AGY28 that contained plasmid pAG7 with the *GAL1-gII*p insert but that lacked the M13 origin within the recombination substrate also did not result in any increase in DEL recombination frequency (Table 2).

To characterize the time course of SSB-induced DEL recombination in dividing cells, cells of strain AGY26 were inoculated into glucose or galactose medium and DEL recombination was determined at different time points. A 4-fold increase in DEL recombination frequency was seen after 8 hr in galactose (Figure 3). After 24 hr growth in galactose, DEL recombination increased about 10-fold (Figure 3). We next investigated the effect of SSBs on DEL recombination frequencies in cell-cycle arrested cells. No significant increase in DEL recombination frequency was seen after exposing to galactose G1-synchronized cells for 8 hr or G2-synchronized cells for 32 hr (Table 3).

One potential reason for a lack of gIIp-induced DEL recombination in arrested cells might be a lack of gIIp expression. Thus, we determined whether exposure of G1-arrested cells to galactose would cause an increase

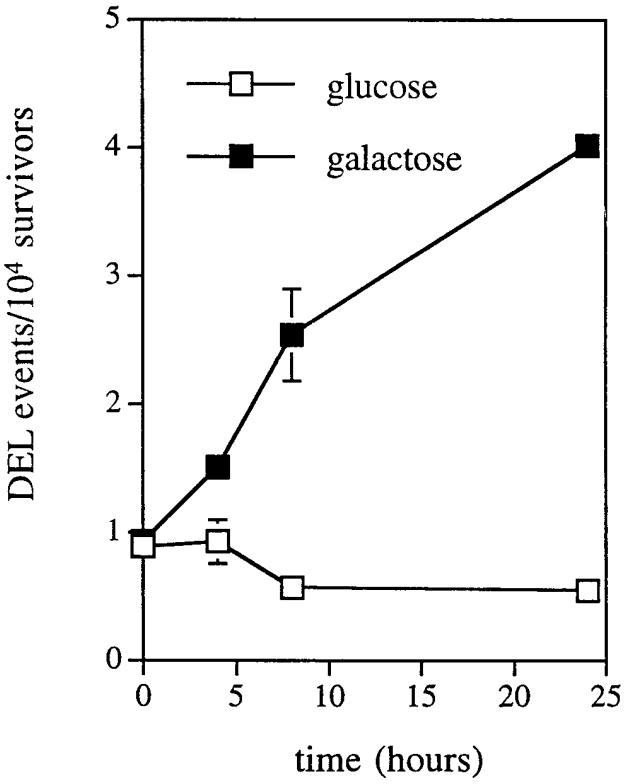

Figure 3.—Effect of gIIp overexpression on DEL recombination in dividing cells. Cells of strain AGY26 were inoculated in SC-ura-leu in the presence of 2% glucose (open rectangles) or 5% galactose (solid rectangles) at time 0. At different times, an aliquot was collected and DEL recombination determined. Results are reported as the mean of three independent experiments ± the standard error.

in DEL recombination in subsequent cell divisions. This was important in order to demonstrate that gIIp was actually making nicks in the DNA of arrested cells. G1-arrested cells were incubated in galactose at the restrictive temperature for 4 hr, then washed and incubated in glucose for 24 hr at the permissive or the restrictive temperatures. An increase in DEL recombination was seen only with cells incubated at the permissive temperature (Figure 4A). No increase in DEL recombination frequency was observed when the cultures were kept under G1 arrest for 24 hr (Figure 4B). This indicates that gIIp is expressed in G1-arrested cells on galactose medium and that the induced nicks cause an increase in DEL recombination frequency in dividing, but not in arrested, cells. Alternatively, a long-lived gIIp protein may be expressed in G1 and nick DNA during subsequent cell divisions.

Effects of UV and γ-rays on DEL recombination frequencies in G1 or G2 cell-cycle-arrested cells and in dividing cells: γ-Rays produce both SSBs and DSBs. DSBs are biologically the most significant lesions. In comparison, UV radiation can produce a variety of photoproducts giving rise to SSBs following repair (Friedberg *et al.* 1995). These two forms of radiation could, therefore,

TABLE 3

Effect of gene II protein overexpression in G1-arrested cells of strain AGY26 and in G2 arrested cells of strain AGY27

A. AGY26 strain:	*CDC28 ura3* cells/10^4 survivors			
Time (hr):	0	2	4	8
Raffinose	3.31 ± 0.37	3.48 ± 0.42	3.44 ± 0.64	4.04 ± 1.44
Glucose	2.38 ± 0.09	2.55 ± 0.37	2.40 ± 0.41	3.57 ± 1.54
Galactose	2.37 ± 0.58	3.18 ± 0.17	3.20 ± 0.33	4.00 ± 0.60

B. AGY27 strain:	*TUB2 ura3* cells/10^4 survivors				
Time (hr):	0	2	6	24	32
Raffinose	0.63 ± 0.11	0.50 ± 0.16	0.51 ± 0.04	0.47 ± 0.05	0.99 ± 0.38
Glucose	0.50 ± 0.20	0.55 ± 0.15	0.58 ± 0.15	0.77 ± 0.29	0.65 ± 0.20
Galactose	0.53 ± 0.01	0.37 ± 0.10	0.51 ± 0.10	0.70 ± 0.25	0.68 ± 0.17

2–3 × 10^7 cells/ml were arrested in G1 (A) or in G2 (B) in SD-ura-leu 2% raffinose and exposed to 2% raffinose, 2% glucose or 5% galactose in G1 (A) or in G2 (B). At 0, 2, 4, and 8 hr (for A) or at 0, 2, 6, 24, and 32 hr (for B) cells were removed washed, and plated. Data are reported as the mean of three independent experiments ± standard error. Results were statistically analyzed using Student's *t*-test.

be used to further investigate the effects of DSBs versus SSBs on DEL recombination in cell-cycle-arrested versus dividing cells. Radiation induction of DEL recombination was measured in cell-cycle-arrested and dividing cells of strain AGY9 and AGY5 for comparison. G1-arrested cells showed no increase in DEL recombination up to UV doses of 100 J/m^2. At 200 J/m^2 and 20% survival, G1-arrested cells showed a weak 2-fold increase in DEL recombination frequency, which was not statistically significant (Table 4A). In contrast, dividing cells

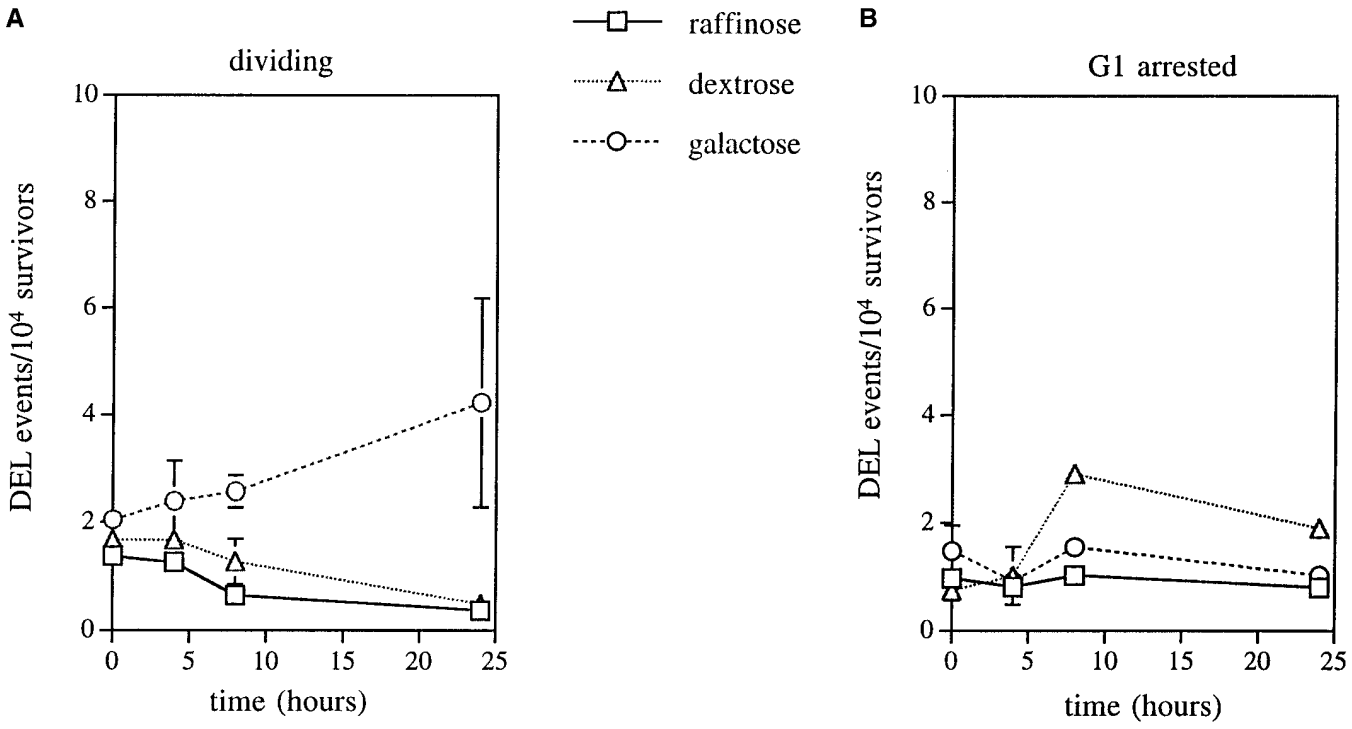

Figure 4.—Effect of cell cycle progression and gIIp expression on DEL recombination in strain AGY26. Cells of AGY26 were arrested in G1 in SC-leu-ura in the presence of raffinose. Then, each culture was divided into three subcultures: one was postincubated in galactose (dashed line and open dots), one in glucose (dotted line and triangle), and one kept in raffinose (solid line and rectangle), but all were kept at the restrictive temperature. After 4 hr, cells were washed and dispensed into glucose-containing medium and each subculture was split in two aliquots; one aliquot was shifted to the permissive temperature to allow cell divisions (A) and the other aliquot was kept arrested in G1 (B). Results are reported as the mean of three independent experiments ± the standard error.

TABLE 4

Induction of DEL recombination by UV and γ-rays in dividing and G1 arrested cells of strain AGY9

	G1-arrested cells			Dividing cells		
	% survival	*CDC28 ura3* cells / 10^4 survivors	Fold increase	% survival	*CDC28 ura3* cells / 10^4 survivors	Fold increase
			A. UV (J/m^2)			
0	100	0.37 ± 0.02	1.0	100	0.38 ± 0.13	1.0
10	66 ± 10	0.26 ± 0.04	0.7	95 ± 1	1.66 ± 0.32	4.4*
50	67 ± 4	0.45 ± 0.11	1.2	72 ± 13	3.54 ± 1.22	9.3*
100	48 ± 3	0.38 ± 0.04	1.0	52 ± 4	8.62 ± 0.25	22.7**
200	20 ± 7	0.75 ± 0.15	2.0	ND	ND	
			B. γ-Rays (Gy)			
0	100	0.49 ± 0.08	1.0	100	0.38 ± 0.13	1.0
10	66 ± 2	1.09 ± 0.25	2.2	ND	ND	ND
50	42 ± 11	1.6 ± 0.37	3.3*	91 ± 9	2.62 ± 0.15	6.9*
100	15 ± 0.3	2.78 ± 0.38	5.7**	89 ± 4	5.06 ± 0.26	13.3**
500	2 ± 0.5	4.09 ± 0.02	8.3***	37 ± 3	10.7 ± 1.0	28.2***
1000	0.2 ± 0.02	36.0 ± 10.0	73.5***	ND	ND	ND

$2–3 \times 10^7$ cells/ml, either arrested or dividing, were exposed to UV and γ-rays as previously reported (Galli and Schiestl 1995a). Then, cells were washed, counted and plated as described in materials and methods. Data are reported as the mean of three independent experiments ± the standard error. Results were statistically analyzed using Student's *t*-test. $*P < 0.05$, $**P < 0.005$, $***P < 0.001$. ND, not determined.

responded with a dose-dependent increase in DEL recombination frequency starting with a statistically significant 4.4-fold increase at the lowest dose of 10 J/m^2 and 95% survival. 100 J/m^2 UV irradiation produced an almost 23-fold increase in DEL recombination frequency in dividing cells (Table 4A). In contrast, γ-rays induced a dose-dependent increase in DEL recombination in both G1-arrested or dividing cells, even at high survival levels (Table 4B). At a dose of 500 Gy, DEL recombination increased 8.3-fold in G1, and 28-fold in dividing cells (Table 4B). G1-arrested cells were less resistant to γ-rays than dividing cells whereas UV irradiation did not result in any difference in survival between G1-arrested and dividing cells (Table 4A).

UV exposure of G2-arrested cells also did not result in any increase in DEL recombination up to 100 J/m^2 and resulted in a barely 2-fold, but significant, increase at 200 J/m^2. UV exposure of dividing cells yielded a dose-dependent increase in DEL recombination with a 3.2-fold significant increase evident even at the lowest dose of 10 J/m^2 (Table 5A). 50 J/m^2 UV increased DEL recombination almost 7-fold in dividing cells while the same UV dose did not cause any increase in G2-arrested cells (Table 5A). γ-Rays again caused a dose-dependent increase in DEL recombination whether cells were arrested in G2 or dividing (Table 5B). G2-arrested cells showed the same sensitivity to UV as dividing cells, while G2 cells were slightly more sensitive to γ-rays than dividing cells (Table 5). In summary, UV irradiation did not induce DEL recombination in cell-cycle-arrested cells until very high doses, whereas it readily induced recombination in dividing cells even at low doses. γ-Rays, on the other hand, induced recombination at low doses in cell-cycle-arrested and dividing cells. These results confirm the above conclusion that DSBs but not SSBs induce DEL recombination in cell-cycle arrested-cells.

Effect of a gIIp-induced SSB after UV or bleomycin exposure on DEL recombination frequencies in cell-cycle-arrested cells: One possible caveat is that the experiments with gIIp expression in cell-cycle-arrested cells do not prove that gIIp is actually making a nick in G1 arrested cells. It is possible that a long-lived gIIp could make the nicks after shift to the permissive temperature. To address this possibility, an additional series of experiments was performed. One way to approach this is to determine DEL recombination frequencies in gIIp overproducing cells arrested in G1 and treated with another SSB producing agent. This treatment could create a second SSB on the DNA strand opposite the gIIp-induced SSB and would result in a DSB causing a synergistic increase in DEL recombination frequency. UV irradiation upon DNA repair (Friedberg *et al.* 1995) and bleomycin (Steighner and Povirk 1990) are agents known to introduce SSBs. Irradiation with 50 J/m^2 of AGY26 cells synchronized in G1 and exposed to galactose for 4 hr resulted in an increase in DEL recombination frequency of 5.2-fold. In comparison, UV irradiation of an aliquot of G1-arrested cells from the same culture, but incubated in raffinose, increased DEL recombination only 2.2-fold and only 1.7-fold in strain AGY26A (w/o M13ori) on galactose (Table 6). Furthermore, exposure of AGY26 cells arrested in G1 to 10 μg/ml bleomycin in galactose medium resulted in a significant 4.3-fold increase in DEL recombination frequency (Table 6). In comparison, exposure to bleomycin of G1-arrested cells of an aliquot from the same

TABLE 5

Induction of DEL recombination by UV and γ-rays in dividing and G2 arrested cells in the strain AGY5

	G2-arrested cells			Dividing cells		
	% survival	*TUB2 ura3* cells/10^4 survivors	Fold increase	% survival	*TUB2 ura3* cells/10^4 survivors	Fold increase
			A. UV (J/m^2)			
0	100	0.63 ± 0.1	1.0	100	0.31 ± 0.12	1.0
10	97 ± 2	0.79 ± 0.08	1.3	88 ± 9	0.99 ± 0.33	3.2*
50	82 ± 8	0.68 ± 0.12	1.1	68 ± 24	2.05 ± 0.40	6.6*
100	69 ± 5	0.84 ± 0.14	1.3	61 ± 17	2.13 ± 0.25	6.9**
200	66 ± 7	1.22 ± 0.16	1.9*	ND	ND	
			B. γ-Rays (Gy)			
0	100	0.25 ± 0.07	1.0	100	0.31 ± 0.12	1.0
10	100	0.38 ± 0.1	1.5	ND	ND	
50	100	0.57 ± 0.29	2.3	98 ± 2	1.54 ± 0.01	5.0**
100	100	0.72 ± 0.12	2.9*	91 ± 9	2.04 ± 0.44	6.6**
500	13 ± 2	2.94 ± 0.32	11.8**	50 ± 14	4.34 ± 0.17	14.0***
1000	9 ± 0.7	6.39 ± 0.35	25.6***	ND	ND	

2–3 × 10^7 cells/ml, either arrested or dividing, were exposed to UV and γ-rays as previously reported (Galli and Schiestl 1995). Then, cells were washed, counted and plated as described in materials and methods. Data are reported as the mean of three independent experiments ± the standard error. Results were statistically analyzed using Student's *t*-test. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$. ND, not determined.

culture in raffinose resulted in only a nonsignificant 1.3-fold increase in DEL recombination frequency. In strain AGY26A (w/o M13ori) on galactose only a nonsignificant 1.6-fold increase was seen (Table 6). In both experiments, G1-arrested cells of strain AGY26 that were not exposed to bleomycin or UV, but incubated on galactose, did not show any induction in DEL recombination frequency. These synergistic increases of DEL recombination frequencies, induced by gIIp expression and additional UV or bleomycin treatment in G1-arrested cells suggest that gIIp does produce SSBs in G1. When these SSBs are combined with another SSB inducing agent, it is likely that the other DNA strand is nicked resulting in a DSB that consequently increases the DEL recombination frequency.

DISCUSSION

This study describes the construction of two genetic systems that select for intrachromosomal recombination events between repeated sequences of *CDC28* and *TUB2* during G1 and G2 cell cycle arrest. Furthermore, we investigated the effects of site-specific DSBs or SSBs between the duplicated alleles on DEL recombination frequencies in dividing and cell-cycle-arrested cells.

Recombination substrates cause cell-cycle arrest: In a previous study we synchronized yeast cells at specific phases of the cell cycle and exposed them to UV and γ-rays (Galli and Schiestl 1995a). Results suggested single-strand annealing or one-sided invasion events were responsible for the majority of induced DEL recombination events. Furthermore, it was suggested that DNA DSBs may be involved in these DEL recombination events (Galli and Schiestl 1995a). This earlier study was hampered by the damage carried over into the next phase of the cell cycle as were several previous studies on DNA damage, repair and recombination processes during cell-cycle arrest. This problem occurs when the cell-cycle block must be released to allow cells to divide to form scorable endpoints (survivors, recombinants, mutants, etc.). Hence long-lived DNA damage may be carried over into the subsequent cell-cycle phase and cause uncertainty as to which specific cell-cycle phase a genetic event occurred in. To avoid this problem, the means to arrest the cells in a specific cell cycle phase was linked with the marker for recombination selection to assure that the induced recombination events actually happen in that particular cell cycle phase. A similar strategy has been previously used by Fabre (Fabre 1978; Fabre *et al.* 1984) to determine the effects of arrest of cells in specific cell-cycle phases on the frequencies and inducibility of interchromosomal recombination.

Induction of DEL recombination by DSBs and SSBs in G1 and G2 versus dividing cells: Several novel isogenic yeast strains allowed the measurement of DEL recombination induced by a site-specific DSB or SSB during cell-cycle arrest. These carry (1) a I-*Sce* I recognition site in intervening DNA sequence between the two repeats of the recombination substrate and (2) the I-*Sce* I nuclease-coding region placed downstream of a *GAL1,10* yeast promoter and integrated in the yeast

TABLE 6

Effect of geneII overexpression on DEL recombination after exposure to UV and bleomycin in G1-arrested cells of strains AGY26A (without gIIp site) and AGY26 (with gIIp site) of *Saccharomyces cerevisiae*

	% survival	DEL events/10^4 surv.	Fold increase
AGY26A			
Galactose	100	5.34 ± 1.61	1
Galactose UV 10 J/m^2	82 ± 6	7.68 ± 2.34	1.4
Galactose UV 50 J/m^2	76 ± 5	9.29 ± 3.58	1.7
Galactose	100	8.17 ± 1.16	1
Galactose Bleo 1μg/ml	87 ± 10	11.47 ± 0.48	1.4
Galactose Beo 10 μg/ml	79 ± 13	12.79 ± 2.55	1.6
AGY26			
Raffinose 2%	100	3.37 ± 0.29	1
Raffinose UV 10 J/m^2	98 ± 2	3.42 ± 0.52	1
Raffinose UV 50 J/m^2	47 ± 3	7.53 ± 0.78*	2.2
Galactose 5%	100	3.17 ± 0.8	1
Galactose UV 10 J/m^2	66 ± 1.5	9.09 ± 1.13*	2.8
Galactose UV 50 J/m^2	30 ± 3.5	16.55 ± 2.59**	5.2
Raffinose 2%	100	2.82 ± 0.15	1
Raffinose Bleo 1 μg/ml	87 ± 3	2.55 ± 0.21	1
Raffinose Bleo 10 μg/ml	58 ± 6	3.82 ± 0.25	1.3
Galactose 5%	100	2.78 ± 0.65	1
Galactose Bleo 1 μg/ml	68 ± 9.2	5.86 ± 0.71*	2.1
Galactose Bleo 10 μg/ml	46 ± 9	12.16 ± 2.43**	4.3

2–3 × 10^7 cells/ml of strain AGY26 were arrested in G1 in medium containing raffinose and lacking uracil and leucine. Aliquots were exposed to galactose for 4 hr and irradiated with UV. For the experiments with bleomycin, the cells were exposed to galactose and the chemical for 8 hr. Thereafter, cells were washed, counted, and plated as described. Data are reported as the mean of three independent experiments ± the standard error. Results were statistically analyzed using Student's *t*-test. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$.

genome. I-*Sce* I generates DSBs in plasmids and chromosomal DNA and thereby increases homologous recombination frequencies (Plessis *et al.* 1992; Fairhead and Dujon 1993). I-*Sce* I induced DSBs also increase recombination frequencies in plant and mouse cells (Puchta *et al.* 1993; Rouet *et al.* 1994). In our experiments I-*Sce* I-generated DSBs induced DEL-recombination frequencies by several hundred- to several thousand-fold, regardless of whether the cells were arrested or dividing. When cells were plated directly onto galactose medium, viability was about 80%, and 99% of all cells underwent DEL recombination events.

To study whether a site-specific SSB increases intrachromosomal DEL recombination we placed the origin of phage-M13 DNA replication in the DNA sequence between the DEL repeats. gIIp produces DNA nicks at its recognition site in the M13 origin (Cleary and Ray 1981). gIIp can stimulate interchromosomal recombination in an interval that contains its recognition site (Strathern *et al.* 1991). The gIIp coding region was placed in a centromeric plasmid under the control of the yeast *GAL1,10* promoter. After 4–5 cell generations in galactose, intrachromosomal DEL recombination frequencies increased 3- to 13-fold. A comparable increase in interchromosomal recombination (5- to 20-fold) was previously observed (Strathern *et al.* 1991). It is possible that a SSB must be transformed into a DSB to increase DEL recombination events. The increase of 3- to 13-fold for intrachromosomal DEL recombination and 5- to 20-fold for interchromosomal recombination (Strathern *et al.* 1991) is modest in comparison to induction of recombination by the HO or the I-*Sce* I enzymes (Rudin and Haber 1988; Nickoloff *et al.* 1989; Fishman-Lobell *et al.* 1992; Plessis *et al.* 1992). In interchromosomal recombination induced by gIIp the gene *II* site preferentially acts as recipient, greater stimulation is observed when the allele nearest the gene *II* site is the defective allele and the gene *II* site is lost in gIIp-stimulated recombination events. These features are in agreement with DSB-induced recombination according to the DSB repair model (Szostak *et al.* 1983) but not with SSB-induced recombination according to the Meselson-Radding model (Meselson and Radding 1975). gIIp causes less than one DSB per 100 SSBs. Strathern *et al.* (1991) proposed that these DSBs induce recombination in their system. It is also worth mentioning that their assay involved dividing cells, thus some SSBs might have developed into DSBs.

In G1- or G2-arrested cells we did not find any induction of DEL recombination by gIIp. There are several explanations for this lack of recombination induction: (1) gIIp might not be active in arrested cells; (2) religa-

tion of the nicks may be more efficient in cell-cycle-arrested cells; (3) the SSBs may have to be converted into DSBs during DNA replication to induce recombination; (4) gIIp may act on a supercoiled substrate (Strathern *et al.* 1991); and (5) replication or DNA damage might be required to make the gIIp site accessible or put it into the proper configuration. Experiments were conducted to address the first two possibilities. In the initial experiment, gIIp was induced in cell-cycle-arrested cells and thereafter cells were shifted to the permissive temperature. Under these conditions gIIp increased the recombination frequency. In a second experiment, cell-cycle-arrested cells were exposed to galactose, to induce gIIp, and at the same time were treated with bleomycin or UV radiation. A synergistic increase in DEL recombination was observed, suggesting that SSBs were indeed generated by gIIp in arrested cells and that additional SSBs were presumably produced by bleomycin or repair of the UV lesions in the opposite DNA strand. It is possible that DSBs are thus created and consequently account for the induced intrachromosomal DEL recombination events. These two experiments suggest that SSBs were actually produced by gIIp but did not induce recombination events in arrested cells.

If gIIp directly induces a certain frequency of DSBs we might have expected an increase in the frequency of DEL recombination in arrested cells. The reason for the lack of any effect may be twofold: (1) the spontaneous frequency might be too high to detect induction by the DSBs and (2) the peculiar structure of the gIIp-induced DSBs might prevent induction. These DSBs have a hairpin loop at one of the two ends of the break (Strathern *et al.* 1991). This structure may prevent degradation of the crosslinked end thus preventing single-strand annealing. During replication, however, DEL recombination may be induced because either these cross-linked strands may be resolved, leading to DSBs accessible to exonucleases, or additional DSBs could be formed from the SSBs.

Effects of UV and ionizing radiation on DEL recombination in G1 and G2 versus dividing cells: G1-, G2-arrested and dividing cells were irradiated with UV and the DEL recombination frequencies determined. UV radiation causes several photoproducts in DNA, including pyrimidine dimers and 6-4 lesions (Friedberg *et al.* 1995). As DNA strand breaks occur only after very high doses of UV exposure, they may not be contributing directly to the biological effects observed after irradiation (Friedberg *et al.* 1995). UV lesions are usually repaired by excision repair and do not lead to DSBs. SSBs, however, occur temporarily during excision repair. Sublethal UV doses induced a large increase of DEL recombination only in dividing cells. For the experiment, cells were harvested from a culture growing in rich medium at the highest growth rate. Under these conditions the S phase occupies 25–50% of the cell cycle (Campbell and Newlon 1991). Thus, the majority of the cells were in S-phase actively replicating their DNA.

There is a cell-cycle checkpoint in the S-phase (Friedberg *et al.* 1995; Kaufmann and Paules 1996) and unrepaired UV lesions and SSBs directly block DNA synthesis. Such unrepaired lesions inhibit initiation of DNA synthesis and also progression of synthesis once DNA replication is already underway (Friedberg *et al.* 1995). Some damaged base pairs are in the form of noncoding lesions, which, as with SSBs, cannot be replicated causing DNA replication to stop. Replication may, though, resume at another replicon downstream. Considering this termination of DNA synthesis in relation to DEL recombination induction, there may be two important consequences. First, such stops in the replication fork could lead to slippage by dissociation of the replicated strand and reassociation with the second homologous copy of the repeat (Bierne and Michel 1994). This alternative mechanism to single-strand annealing would involve no strand breakage and rejoining but does require DNA replication. Second, it has been proposed that DNA adducts and SSBs could be converted to replicative gaps, and, finally, DSBs, whenever these lesions were carried into the S-phase (Kaufmann and Paules 1996). Moreover, it has been suggested that UV-induced damage can persist in the DNA, through several cell divisions, to finally produce a chromosomal aberration likely caused by a DSB (Galloway 1994). It is not clear though, exactly how the UV-induced DNA damage might be transformed into a DSB. When incision of a pyrimidine dimer cannot occur, as in a *rad1* mutant, the frequency of sister-chromatid recombination increases (Brown *et al.* 1991). When UV lesions are maintained in the genomic DNA for several generations, as in incision deficient mutants (Fabre 1981), many DSBs could be produced by DNA replication. Irradiation of excision-repair-competent cells in the S-phase may mimic this situation by restricting repair of the lesion to a time before replication of the damaged DNA ensues. In view of these observations, the induction of DEL recombination by UV in dividing cells may be explained by replication on the damaged template, which could take place in cells before the completion of DNA synthesis. High doses of UV exposure, however, did weakly induce DEL recombination in arrested cells. This may be explained by the creation of DSBs by excision-repair tracts in close proximity but on opposite DNA strands.

Ionizing radiation, such as γ-rays, induces many lesions with DSBs having the most significant biological effect (Friedberg *et al.* 1995). Sublethal γ-ray doses induced DEL recombination in both cell-cycle-arrested and dividing cells. This further suggested that DSBs are responsible for the induction of DEL recombination. DNA-DSBs in yeast are repaired mainly by homologous recombination (Game 1993). γ-Rays induced DEL recombination to the same extent in G1 and G2, and to

a higher extent in dividing cells. This higher extent could be due to other DNA lesions including SSBs and base damage being converted to DSBs in dividing cells. One Gy of ionizing radiation causes 16 to 40 DSBs, 600 to 1000 SSBs, and 250 damaged-thymine residues in DNA (Ward 1988). An alternative explanation is that such DNA damage might induce template switching, after DNA polymerase stops in front of the damage and subsequent translocation and reannealing of the newly replicated strand to the homologous sequence as described above.

Kadyk and Hartwell (1993) studied UV-induced sister-chromatid recombination in *rad1* mutants. In *rad1* mutants, UV irradiation in G1 induces sister-chromatid recombination in the subsequent G2 phase. The authors proposed either that unexcised dimers induce sister-chromatid recombination during replication as a mechanism to repair lesions or that strand breaks created directly by X rays or excision of UV-damaged bases give rise to the recombinagenic substrates. We found a dependence of UV-induced DEL recombination on cell division and thus probably DNA replication. Since deletions and duplications of the integrated plasmid would be reciprocal products of SCE, it would be tempting to explain our results in a similar way. However, the following differences should be kept in mind. First, the frequencies of spontaneous recombination with the two endpoints are about 100-fold different (about 2×10^{-6} for sister-chromatid recombination (Fasullo and Davis 1987; Kadyk and Hartwell 1992) versus about 2×10^{-4} for DEL recombination) making it less likely that they are reciprocal products of the same event. Furthermore, UV irradiation exclusively induced conversion events (Kadyk and Hartwell 1993), which may be caused by strand breaks. Thus, the second explanation that recombinagenic strand breaks (DSBs in our case) are created is a more likely explanation for our events.

Interchromosomal recombination is inducible to much higher levels by ionizing and UV radiation in G1 than in G2 (Esposito 1968; Fabre *et al.* 1984). This indicates that in G2, DNA strand breaks are preferentially repaired by sister-chromatid recombination (Brunborg *et al.* 1980; Kadyk and Hartwell 1992). γ-Rays, however, induced DEL recombination to the same extent in G1 and in G2, suggesting that sister-chromatid recombination does not account for the majority of γ-ray induced DEL recombination events.

It has been previously shown that interchromosomal recombination is well inducible by UV in G1 (Fabre 1978; Fabre *et al.* 1984) whereas DEL recombination events in our present study were not. In previous experiments we arrested cells at the *cdc28* block and scored DEL recombination at *HIS3* and interchromosomal recombination at *ADE2* (Galli and Schiestl 1995a). In G1-arrested cells we found that DEL recombination was highly inducible by ionizing radiation but only very weakly inducible by UV. In contrast, interchromosomal recombination was highly inducible by ionizing radiation as well as UV. Thus, the differences between the two results are likely due to the different endpoints (interchromosomal recombination versus intrachromosomal DEL recombination events).

Because SSBs are relatively harmless lesions and are repaired with greater efficiency than DSBs it was not too surprising that SSBs in the nonhomologous region between duplicated sequences did not induce recombination. A total of 400,000 H_2O_2-induced SSBs, compared to only 40 DSBs, produce one lethal hit in mammalian cells (Ward *et al.* 1987). What is shown in this article, however, is that this relatively harmless DNA damage turns into a recombinagenic lesion upon DNA replication. This transformation of lesions is important for carcinogenesis and hence the importance of DNA-damage-induced cell-cycle control blocks in G2 and G1 (Friedberg *et al.* 1995; Hartwell *et al.* 1994; Schiestl *et al.* 1989; Siede *et al.* 1993; Weinert and Hartwell 1988). Such blocks allow time for DNA repair and prevent creation of more harmful DNA damage by replication. In fact, chemical carcinogenesis and transformation are most efficient if the target cells are treated just prior or during S-phase (see citations in Friedberg *et al.* 1995; Kaufmann and Paules 1996). Thus, DNA replication turns relatively harmless DNA damage into recombinagenic and carcinogenic lesions.

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