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A Mutant Allele of the Transcription Factor IIH Helicase Gene, *RAD3*, Promotes Loss of Heterozygosity in Response to a DNA Replication Defect in *Saccharomyces cerevisiae*

Michelle S. Navarro,* Liu Bi† and Adam M. Bailis*,[1]

**Division of Molecular Biology, Beckman Research Institute, City of Hope National Medical Center, Duarte, California 91010-0269 and* †*Department of Botany and Plant Sciences, University of California, Riverside, California 92521*

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ABSTRACT

Increased mitotic recombination enhances the risk for loss of heterozygosity, which contributes to the generation of cancer in humans. Defective DNA replication can result in elevated levels of recombination as well as mutagenesis and chromosome loss. In the yeast *Saccharomyces cerevisiae*, a null allele of the *RAD27* gene, which encodes a structure-specific nuclease involved in Okazaki fragment processing, stimulates mutation and homologous recombination. Similarly, *rad3-102*, an allele of the gene *RAD3*, which encodes an essential helicase subunit of the core TFIIH transcription initiation and DNA repairosome complexes confers a hyper-recombinagenic and hypermutagenic phenotype. Combining the *rad27* null allele with *rad3-102* dramatically stimulated interhomolog recombination and chromosome loss but did not affect unequal sister-chromatid recombination, direct-repeat recombination, or mutation. Interestingly, the percentage of cells with Rad52-YFP foci also increased in the double-mutant haploids, suggesting that *rad3-102* may increase lesions that elicit a response by the recombination machinery or, alternatively, stabilize recombinagenic lesions generated by DNA replication failure. This net increase in lesions led to a synthetic growth defect in haploids that is relieved in diploids, consistent with *rad3-102* stimulating the generation and rescue of collapsed replication forks by recombination between homologs.

GENOMIC integrity and, ultimately, cell survival rely on the coordinated and accurate responses of various damage repair systems to insults incurred by the DNA. In their absence, chromosomal instability, a hallmark of tumor cells, is markedly increased (Mitelman *et al.* 1994; Radford *et al.* 1995; Gupta *et al.* 1997; Lengauer *et al.* 1998; Gray and Collins 2000; Bishop and Schiestl 2001; Feitelson *et al.* 2002; Kamb 2003; Lin *et al.* 2003; Rajagopalan and Lengauer 2004). Homologous recombination (HR) is a repair mechanism that is critical for repairing double-strand breaks (DSBs) created by DNA replication failure, ionizing radiation, and other damaging agents (Game and Mortimer 1974; Resnick 1976; Resnick and Martin 1976; Tishkoff *et al.* 1997; Symington 1998; Paques and Haber 1999; Cox *et al.* 2000; Debrauwere *et al.* 2001; Galli *et al.* 2003; Michel *et al.* 2004). Many of the genes involved in HR, such as *RAD50*, *RAD51*, *RAD52*, *RAD53*, *RAD54*, *RAD55*, *RAD56*, *RAD57*, *RAD59*, *RDH54/TID1*, *MRE11*, and *XRS2* (*NBS1* in humans), were first identified through mutants sensitive to ionizing radiation (Game and Mortimer 1974). The HR proteins physically interact with and process DSBs to facilitate their repair (Paques and Haber 1999; Sugawara *et al.* 2003; Krogh and Symington 2004).

[1]*Corresponding author:* Division of Molecular Biology, Beckman Institute of the City of Hope, 1450 E. Duarte Rd., Duarte, CA 91010-0269. E-mail: abailis@bricoh.edu

Repair by HR requires an initiating event, such as a DSB (Resnick 1976; Resnick and Martin 1976; Szostak *et al.* 1983; Paques and Haber 1999), and a homologous donor sequence carrying sufficient genetic information to repair the break (Rubnitz and Subramani 1984; Bailis and Rothstein 1990; Sugawara and Haber 1992; Jinks-Robertson *et al.* 1993). The donor sequences most commonly used to repair DSBs are homologous sequences on the sister-chromatid or homologous chromosome. However, increased mitotic recombination with a homologous chromosome or nonallelic, ectopic sequences increases the risk for deleterious genome rearrangements or loss of heterozygosity (LOH) and paves the road for carcinogenesis or other diseases (Gupta *et al.* 1997; Bishop and Schiestl 2001). Several studies have demonstrated that the development of many cancers involves the loss or gain of information by interhomolog recombination mechanisms such as gene conversion and break-induced replication (Bishop and Schiestl 2001). For example, one study reported that 81.3% of colorectal adenocarcinomas exhibited LOH (Lin *et al.* 2003), while, in another study, up to 37.5% of ductal carcinoma *in situ* of the breast displayed LOH (Radford *et al.* 1995).

Identifying the factors that stimulate interhomolog recombination may provide insight into the molecular mechanisms that promote LOH and cell transformation. Defects in DNA replication proteins such as Dna2,

Pol δ, and Rad27 stimulate mitotic recombination (Symington 1998; Aguilera *et al.* 2000; Lopes *et al.* 2002; Michel *et al.* 2004), perhaps by interfering with DNA replication fork progression. Alternatively, a DNA replication fork may encounter a lesion that blocks leading- or lagging-strand synthesis (Holmes and Haber 1999; Lopes *et al.* 2006b). Lesions can be bypassed using error-prone mechanisms or through template switching to the sister chromatid. If the lesion persists, leaving a daughter-strand nick or gap (Lopes *et al.* 2006b), subsequent DNA replication may generate a collapsed fork that resembles a single-ended DSB (Saleh-Gohari *et al.* 2005; Cortes-Ledesma and Aguilera 2006). Whereas mutagenic bypass and template switching may aid in preventing fork collapse and DSB formation, the requirement for HR to rescue collapsed forks is clearly suggested by the observation that certain DNA replication mutations are lethal in combination with mutations in the HR machinery (Tishkoff *et al.* 1997; Symington 1998; Debrauwere *et al.* 2001; Galli *et al.* 2003; Michel *et al.* 2004).

In yeast, the *RAD27* gene codes for a 5′–3′ flap exo- and endonuclease that processes Okazaki fragments during lagging-strand DNA synthesis and is the homolog to the human FEN-1 protein (Harrington and Lieber 1994; Reagan *et al.* 1995; Sommers *et al.* 1995; Lieber 1997). In the *rad27*-null mutant, all consequences of defective lagging-strand synthesis are observed. For example, *rad27*-null mutant cells display increased levels of single-stranded DNA (Vallen and Cross 1995; Parenteau and Wellinger 1999), mutagenesis (Tishkoff *et al.* 1997), microsatellite instability (Schweitzer and Livingston 1998; Xie *et al.* 2001; Refsland and Livingston 2005), minisatellite instability (Kokoska *et al.* 1998; Lopes *et al.* 2006a), telomeric repeat instability (Parenteau and Wellinger 1999), and recombination (Tishkoff *et al.* 1997; Symington 1998; Negritto *et al.* 2001). Combining the *rad27*Δ-null allele with null mutations in a large number of HR genes leads to synthetic lethality, strongly suggesting the need for HR to rescue DNA replication defects in these cells (Tishkoff *et al.* 1997; Symington 1998; Debrauwere *et al.* 2001).

Previous studies in yeast showed that certain alleles of the *RAD3*, *SSL1*, and *SSL2* genes, which encode subunits of the transcription factor IIH (TFIIH) and nucleotide excision repair (NER) complexes, display elevated levels of mitotic recombination (Golin and Esposito 1977; Malone and Hoekstra 1984; Montelone *et al.* 1988, 1992; Montelone and Liang-Chong 1993; Montelone Bailis *et al.* 1995; Bailis and Maines 1996; Garfinkel and Bailis 2002). This suggests a role for these DNA repair genes either in preventing the formation of lesions that utilize recombination for repair or in modulating the recombinational response to these lesions. TFIIH and the NER repairosome share seven subunits that include *RAD3*, *SSL1*, *SSL2*, *TFB1*, *TFB2*, *TFB4*, and *TFB5* (Svejstrup *et al.* 1995; Feaver *et al.* 2000; Takagi *et al.* 2003; Ranish *et al.* 2004). TFIIH is required for transcription initiation by RNA polymerase II, while the NER repairosome is required for the repair of UV damage (Feaver *et al.* 1993; Svejstrup *et al.* 1995). Both *RAD3* and *SSL2* code for DNA-dependent ATPase/helicases with opposite polarities and are essential for cell viability (Higgins *et al.* 1983; Naumovski and Friedberg 1983; Sung *et al.* 1987; Guzder *et al.* 1994; Sung *et al.* 1996). Recently, the Ssl1 subunit has also been shown to possess E3 ubiquitin ligase activity (Takagi *et al.* 2005). Since Ssl1 and Rad3 interact physically and genetically (Bardwell *et al.* 1994; Maines *et al.* 1998), it is possible that this function may be integral to the roles already described for these proteins.

Mutant alleles of the *RAD3*, *SSL1*, and *SSL2* genes confer several genetically separable phenotypes, indicative of their roles in multiple cellular functions. Consistent with these observations, human homologs of these genes have been linked to the diseases xeroderma pigmentosum, Cockayne's syndrome, and trichothiodystrophy, which are characterized by transcriptional and DNA repair defects and increased tumor formation (Garfinkel and Bailis 2002). While mutations in the yeast *RAD3* and *SSL1* genes create separable transcription, NER, and recombination phenotypes (Montelone *et al.* 1988; Song *et al.* 1990; Feaver *et al.* 1993; Montelone and Malone 1994; Wang *et al.* 1995; Maines *et al.* 1998), it remains unclear how these changes are related.

One *RAD3* mutant allele, *rad3-102*, was identified on the basis of its ability to confer elevated levels of mitotic recombination and mutagenesis (Malone and Hoekstra 1984). The *rad3-102* allele contains a point mutation that alters amino acid residue 661 in the seventh conserved domain of the Rad3 helicase (Montelone and Malone 1994). Further, like the *rad27*-null allele, *rad3-102* confers synthetic lethality when combined with mutations in the HR genes *RAD52* and *RAD50* (Malone and Hoekstra 1984). This may be independent from the transcription initiation and NER function of Rad3 as the *rad3-102* mutation does not confer any apparent transcription defect, and only a minor NER defect (Hoekstra and Malone 1987; Malone *et al.* 1988). In this article, we examine the epistatic interactions between *rad3-102* and a null allele of *RAD27* to determine if their elevated rates of mutation and recombination are mechanistically related.

MATERIALS AND METHODS

Yeast strains and plasmids: All strains used in this study are isogenic with W303-1A (Thomas and Rothstein 1989) and are shown in Table 1. All yeast strain construction used conventional methods (Burke *et al.* 2000). The construction of the *rad27::LEU2* allele was described previously (Frank *et al.* 1998) and the *rad3-102* allele was incorporated into yeast from the plasmid pBM3-102 that was a generous gift of Beth Montelone and Robert Malone (Montelone *et al.* 1988;

TABLE 1

Yeast strains used in this study

Strain	Genotype[a]	Reference
ABX471-2C	*MAT*α *hom3-10 lys2-Bgl CAN1*	This study
ABX474-4B	*MAT*α *hom3-10 lys2-Bgl CAN1 rad27::LEU2*	This study
ABX460-6A	*MAT***a** *hom3-10 lys2-Bgl, CAN1 rad3-102*	This study
ABX481-1C	*MAT*α *hom3-10 lys2-Bgl CAN1 rad27::LEU2 rad3-102*	This study
ABX761	*MAT***a**/α *CAN1/can1-100 his3-Δ200/HIS3 trp1-1/trp1-1::his3-Δ3′::his3-Δ5′::URA3 rad3-102/RAD3 rad27::LEU2/RAD27*	This study
ABX1465	*MAT***a**/α *his3::URA3::his3/HIS3 rad3-102/RAD3*	This study
ABX1368	*MAT***a**/α *his3Δ-200/his3::URA3::his3 TRP1/trp1-1 rad27::LEU2/RAD27*	This study
ABX1308	*MAT***a**/α *CAN1/can1-100 his3Δ-200/his3::URA3::his3 rad27::LEU2/RAD27 rad3-102/RAD3*	This study
ABX633	*MAT***a**/α *can1-100/CAN1 hom3-10/HOM3 HIS3/his3-11, 15, trp1-1/TRP1, URA3/ura3-1 LYS2/lys2Δ-Bgl*	This study
ABX647	*MAT***a**/α *can1-100/CAN1 HOM3/hom3-10 HIS3/his3::ura3::LEU2 TRP1/trp1-1 rad3-102/rad3-102*	This study
ABX658	*MAT***a**/α *can1-100/CAN1 HOM3/hom3-10 TRP1/trp1-1 his3::URA3::his3/HIS3 rad27::LEU2/rad27::LEU2*	This study
ABX693	*MAT***a**/α *can1-100/CAN1 HOM3/hom3-10 ura3-1/URA3 LYS2/lys2ΔBgl rad3-102/rad3-102 rad27::LEU2/rad27::LEU2*	This study
ABX861	*MAT***a**/α *ADE2/ade2-1 his3::URA3::his3/HIS3 rad3-102/RAD3 rad27::LEU2/RAD27 RAD52/RAD52-YFP*	This study
W961-5A	*MAT***a** *HIS3*	John McDonald
ABX362-14C	*MAT***a** *rad3-102*	This study
ABX217-3C	*MAT***a** *HIS3 rad27::LEU2*	This study
ABX397-3A	*MAT***a** *rad3-102 rad27::leu2::hisG*	This study
ABX447	*MAT***a**/α *leu2-3, 112 RAD27/rad27::LEU2*	This study
ABX430	*MAT***a**/α *rad3-102/rad3-G595R*	This study
ABX1869	*MAT***a**/α *ADE2/ade2-1, CAN1/can1-100, his3-11, 17/HIS*3, TRP1/trp1-1, *leu2-3, 112 rad27::LEU2/RAD27 RAD3/rad3-102*	This study
ABT576	*MAT*α, *LEU2*, pRS416 (*URA3*)	This study
ABT577	*MAT*α, *LEU2*, pJM3 (*MAT***a**, *URA3*)	This study
ABT581	*MAT*α, *HIS3, rad27::LEU2, rad3-102,* pRS416 (*URA3*)	This study
ABT582	*MAT*α, *HIS3, TRP1, rad27::LEU2, rad3-102,* pJM3 (*MAT***a**, *URA3*)	This study
ABX1358	*MAT***a**/α *his3::URA3::his3/his3-Δ200, TRP1/trp1-1*	This study
ABX1369	*MAT***a**/α *his3::URA3::his3/his3-Δ200, TRP1/trp1-1, rad3-102/rad3-102*	This study
ABX1362	*MAT***a**/α *his3::URA3::his3/his3-Δ200, TRP1/trp1-1, rad27::LEU2/rad27::LEU2*	This study
ABX2010	*MAT***a**/α *his3::URA3::his3/his3-Δ200, TRP1/trp1-1, rad27::LEU2/rad27::LEU2, rad3-102/rad3-102*	This study
ABX1498	*MAT***a**/α *can1-100/CAN1, hom3-10/HOM3, ura3::KANMX/ura3::KANMX, HXT13/hxt13::URA3*	This study
ABX1204	*MAT***a**/α *can1-100/CAN1, hom3-10/HOM3, ura3::KANMX/ura3::KANMX, HXT13/hxt13::URA3, rad3-102/rad3-102*	This study
ABX1175	*MAT***a**/α *can1-100/CAN1, hom3-10/HOM3, ura3::KANMX/ura3::KANMX, HXT13/hxt13::URA3, rad51::LEU2/rad51::LEU2*	This study
ABX1611	*MAT***a**/α *can1-100/CAN1, hom3-10/HOM3, HIS3/HIS3, ura3::KANMX/ura3::KANMX, HXT13/hxt13::URA3, rad51::LEU2/rad51::LEU2, rad3-102/rad3-102*	This study

[a] All strains were isogenic to W303-1A (*MAT***a** *ade2-1 can1-100 his3-11,17 leu2-3,112 trp1-1 ura3-1 rad5-G535R*) (Thomas and Rothstein 1989). Only deviations from this genotype are listed.

Montelone and Malone 1994). All strains derived from those containing the *rad27::LEU2* mutation were checked for differences in growth to assure absence of suppressors. The *rad27*-null mutation confers a slow-germination phenotype that is easily distinguishable from wild-type strains. ABX761, used for the unequal sister-chromatid recombination (USCR) assay, was constructed by transforming *Xba*I-digested PNN287, the generous gift of Michael Fasullo (Fasullo and Davis 1987), into ABX731-8C (*MAT***a**, *his3-Δ200*) by electroporation. Insertion of the plasmid at the targeted site was verified by Southern blot analysis (M. S. Navarro and A. M. Bailis, unpublished results). Yeast strains containing the *hom3-10, lys2-Bgl*, and *CAN1* alleles were derived from strain RKY2672 from Richard Kolodner (Tishkoff *et al.* 1997) and backcrossed into our background at least four times. The *RAD52-YFP* allele was the generous gift of Michael Lisby and Rodney Rothstein (Lisby *et al.* 2001). Assays were conducted from haploids taken immediately from dissection plates or from diploids constructed from newly dissected spore colonies.

The impact of the *rad5-G535R* allele on growth, mutation, recombination, and chromosome loss was shown to be minimal and did not change the effects exerted by the *rad3-102* or

rad27-null alleles singly or in combination (M. S. Navarro and A. M. Bailis, unpublished results).

Centromere-containing plasmid pRS416 (Christiansen *et al.* 1991), which contains the *URA3* gene, and pJM3, which contains the *URA3* and *MAT***a** genes, were generously provided by Phil Hieter and Jim Haber.

Determination of mutation rates: The *lys2-Bgl*, *hom3-10*, and *CAN1* mutation rates for ABX471-2C (wild type), ABX474-4B (*rad27*), ABX460-6A (*rad3-102*), and ABX481-1C (*rad27*Δ *rad3-102*) were determined as described previously (Tishkoff *et al.* 1997). Strains were plated on YPD (2% peptone, 1% yeast extract, 2% dextrose) for single colonies at 30°. For each genotype, seven individual colonies from at least five strains were excised from plates and individually suspended in sterile water. Appropriate dilutions were plated onto YPD to determine the total number of viable cells, onto synthetic medium lacking lysine to determine the number of lysine prototrophic (Lys^+) cells per colony, onto synthetic medium lacking threonine to determine the number of threonine prototrophic (Thr^+) cells per colony, and onto synthetic medium without arginine plus 60 μg/ml of canavanine to determine the number of canavanine resistant (Can^r) cells per colony. Individual rates were calculated using the method of the median (Lea and Coulson 1949) and are expressed as the number of mutation events/cell/generation. Confidence intervals of 95% were determined by a previously described method (Spell and Jinks-Robertson 2004). A minimum of 30 cultures were tested per strain.

Determination of unequal sister-chromatid recombination rates: The rates of unequal sister-chromatid recombination were determined as previously described (Fasullo and Davis 1987). At least five freshly dissected ABX761 segregants (of each genotype) containing the USCR construct and the *his3-Δ200* allele were streaked out for single colonies on YPD. After 3 days of growth at 30°, at least three single colonies were excised from each plate and suspended in sterile water. Appropriate dilutions were plated onto YPD to determine the total number of viable cells and synthetic complete medium lacking histidine to determine the number of His^+ cells per colony. After growth at 30° for 3–4 days, the number of His^+ events was determined. Unequal sister-chromatid recombination rates and 95% confidence intervals were calculated from a minimum of 15 trials as described above.

Determination of direct-repeat recombination rates: The direct-repeat assay was performed as described previously (Maines *et al.* 1998). Two truncated *HIS3* sequences, sharing 415 bp of homology, flank a *URA3* marker at the *HIS3* locus on chromosome XV. Recombination between the duplicate sequences results in deletion of the *URA3* marker and generation of a wild-type *HIS3* allele. At least 15 freshly dissected segregants containing the direct repeat were grown to saturation in synthetic medium lacking uracil at 30°. Appropriate dilutions of cells were plated onto YPD medium to determine the number of viable cells and onto synthetic complete medium lacking histidine to determine the number of His^+ recombinants. Plates were incubated at 30° for 4 days. Deletion rates and 95% confidence intervals were calculated from a minimum of 15 trials as described above.

Determination of chromosome V loss and interhomolog recombination rates: The rates of interhomolog recombination (IHR) and chromosome V loss were determined as described previously (Klein 2001). Freshly dissected segregants containing either *CAN1* and *HOM3* or *can1-100* and *hom3-10* alleles were crossed to generate *CAN1/can1-100*, *HOM3/hom3-10* diploid strains. At least five independent diploids of each genotype were prepared. Seven fresh colonies of each diploid were dispersed in sterile water. Appropriate dilutions of cells were plated onto YPD medium to determine the total number of viable cells and onto synthetic medium lacking arginine and containing 60 μg/ml of canavanine to determine the number of Can^r colonies. The total number of Can^r colonies was determined after growth at 30° for 4 days. Can^r colonies were replica plated onto synthetic medium lacking threonine. After 2 days of growth at 30°, the fractions of colonies that were Can^r Thr^+ and Can^r Thr^- were determined. Cells that were Can^r Thr^- were scored as chromosome loss events and cells that were Can^r Thr^+ were scored as interhomolog recombination events. Can^r Thr^+ colonies may also represent mutation events; however, it was found that Can^r Thr^+ colonies result predominantly from mitotic recombination (Golin and Esposito 1977). Rates and 95% confidence intervals were determined from a minimum of 35 trials as described above.

To distinguish break-induced replication events from gene conversion events among interhomolog recombination events, we inserted the *URA3* marker within the telomere proximal *hxt13* locus on chromosome V as previously described (Chen and Kolodner 1999). For the assay, the *hxt13::URA3* allele is on the same copy of chromosome V copy that contains the wild-type *HOM3* and *CAN1* alleles. Appropriate dilutions of diploid cells were plated onto YPD medium and onto synthetic medium lacking arginine and containing canavanine to determine the number of Can^r colonies. The total number of Can^r colonies was determined after growth at 30° for 4 days. Can^r colonies were replica plated onto synthetic medium lacking threonine and onto synthetic medium lacking uracil. After 2 days of growth at 30°, the fractions of colonies that were Can^r Thr^+ Ura^+ were scored as gene conversion events, cells that were Can^r Thr^+ Ura^- were scored as break-induced replication events, and cells that were Can^r Thr^- Ura^- were scored as chromosome loss events. It is important to note that we were unable to distinguish between break-induced replication (BIR) and crossover events with this assay.

Fluorescence microscopy: All experiments were performed according to previously described methods (Lisby *et al.* 2001). In brief, 5 ml of cells were grown in complete synthetic medium to an OD_{600} of 0.2 at room temperature. Growth at room temperature is required to allow the chromophore to form efficiently (Lim *et al.* 1995). One milliliter of cells was then washed and resuspended in 200–300 μl of complete synthetic medium. A small aliquot of cells was placed on a glass slide and sealed with VALAP solution (a combination of equal volumes of petroleum jelly, lanolin, and paraffin). Cells were visualized using an Olympus (Melville, NY) AX70 automated upright microscope containing a mercury illumination source and a U-MWIBA filter cube (excitation 460–490 nm) for visualizing Rad52-YFP. Ten live cell images were taken at 0.1-μm intervals along a *z*-axis, using a Spot RT slider high-resolution B/W camera and a Plan/Apochromat 60x, 1.4 numerical aperture lens, and prepared using the Image Pro Plus software (Media Cybernetics, Silver Spring, MD) and Image J software (National Institutes of Health, Bethesda, MD). Brightfield images were used to count total cell number and define cell-cycle phase, and each *z*-stack fluorescence image was inspected for the presence of a Rad52-YFP focus.

Determination of doubling time: YPD liquid (5 ml) was inoculated with a single colony and grown overnight at 30°. Aliquots from each wild-type, *rad3-102*, *rad27*-null, and *rad3-102 rad27*-null culture were used to inoculate 5 ml of YPD to a cell density of $\sim 1 \times 10^7$ cells/ml and grown at 30°. Culture density was measured each hour by monitoring turbidity using a Klett–Summerson colorimeter fitted with a red filter. Doubling times were calculated using a common algorithm (Singleton 1995). Growth assays with strains containing either pJM3 or pRS416 were done using synthetic complete medium lacking uracil to maintain selection for the plasmids.

TABLE 2

Mutation rate analysis in wild-type and mutant strains

Strain	Genotype	Mutation rate		
		Can^r ($\times 10^{-7}$)	Hom$^+$ ($\times 10^{-7}$)	Lys$^+$ ($\times 10^{-7}$)
ABX471-2C	Wild type	4.0 (3.8–4.4)	0.26 (0.21–0.29)	0.32 (0.27–0.42)
ABX460-6A	*rad3-102*	5.7 (4.8–7.5)	1.9 (1.6–2.6)	2.6 (1.9–3.2)
ABX474-4B	*rad27*	64 (54–88)	2.2 (1.3–3.0)	5.3 (3.6–7.6)
ABX481-1C	*rad3-102 rad27*	69 (47–85)	1.7 (0.85–2.0)	4.0 (2.9–4.8)

All rates were determined from a minimum of 30 trials as described in MATERIALS AND METHODS; 95% confidence intervals are indicated in parentheses.

RESULTS

Mutagenic response to DNA replication defects is unaltered in the *rad3-102 rad27*-null double mutant: The *rad27*-null mutant confers a defect in lagging-strand synthesis that was previously shown to increase mutagenesis in several mutation assays (TISHKOFF *et al.* 1997). Since the *rad3-102* mutant also displays an increased mutation rate (MALONE and HOEKSTRA 1984; MONTELONE *et al.* 1992; MONTELONE and LIANG-CHONG 1993; MONTELONE and KOELLIKER 1995), we investigated the epistatic relationship between the *rad27*-null and *rad3-102* mutations with respect to mutagenesis. The *CAN1* forward mutation assay and the *hom3-10* and *lys2-Bgl* reversion assays previously utilized to characterize the *rad27*-null mutant were used (TISHKOFF *et al.* 1997). The *CAN1* forward mutation rate assay selects for cells made resistant to canavanine (Can^r) by mutagenesis of the arginine permease gene (GRENSON *et al.* 1966). The reversion assays select for revertants of either a 4-base insertion in the *LYS2* gene (*lys2-Bgl*) or a +1 T insertion within a run of six T's in the *HOM3* gene (*hom3-10*). Consistent with previous results (TISHKOFF *et al.* 1997), we observed significant increases in mutation rate with all three assays in the *rad27*-null mutant cells (Table 2): a 16-fold increase in the rate of *CAN1* mutation, an 8-fold increase in the rate of *hom3-10* reversion, and a 17-fold increase in the rate of *lys2-Bgl* reversion. *rad3-102* cells displayed significantly increased rates with only the *hom3-10* (7-fold increased) and *lys2-Bgl* (8-fold increased) reversion assays. Interestingly, *rad3-102* did not alter the mutator effect of the *rad27*-null mutation, even in the *hom3-10* reversion assay where its stimulation is similar to that seen with the *rad27*-null mutation. These results suggest that the *rad3-102* and *rad27*-null mutations affect the same mutagenic mechanism. To further confirm this conclusion, we determined the spectrum of representative *lys2-Bgl* reversion mutations from wild-type, *rad3-102*, *rad27*-null, and *rad3-102 rad27*-null double mutants. Sequencing of nucleotides 315–540 of the *LYS2* gene from at least 20 independent Lys$^+$ revertants revealed that the mutation spectrum of the *rad3-102 rad27*-null double mutant was similar to that of the *rad27*-null single mutant, particularly with regard to the deletion/insertion mutations that are characteristic of *rad27*-null mutant cells (44 and 36%, respectively). No deletion/insertion mutations appeared in wild-type or *rad3-102* Lys$^+$ revertants (L. BI and A. M. BAILIS, unpublished results).

Unequal sister-chromatid and direct-repeat recombination are unaffected by the *rad3-102* allele in the *rad3-102 rad27*-null double mutants: Template switching with the sister chromatid is another response to replication lesions (DONG and FASULLO 2003). Consequently, we investigated the epistatic relationship between the *rad3-102* and *rad27*-null mutations with respect to USCR using an assay developed by FASULLO and DAVIS (1987). The assay monitors the frequency of recombination between 3′ and 5′ truncated copies of the *HIS3* gene that share 300 bp of homologous sequence and are arranged tail to head at the *TRP1* locus on chromosome IV. This arrangement restricts the production of a functional *HIS3* gene until S-phase. The rate of USCR in the *rad27*-null single mutant was 48-fold over the wild-type rate and the *rad3-102* mutant increased recombination <4-fold over wild type (Table 3). USCR in the *rad3-102 rad27*-null double mutant was not significantly different from the *rad27*-null single mutant.

Unlike USCR, deletion formation by recombination between nontandem direct repeats on a chromosome can occur before or after the initiation of replication. We determined the rates of recombination between 3′ and 5′ truncated copies of the *HIS3* gene that share 415 bp of homology and flank a *URA3* selectable marker in haploid and diploid cells (MAINES *et al.* 1998). The rates of direct-repeat recombination in haploid cells were increased sevenfold in the *rad27*-null mutant and only twofold in the *rad3-102* mutant (Table 3). Like USCR, the direct-repeat recombination rate in double-mutant haploids was not significantly different from the rate in *rad27*-null single mutants, although the possible presence of the additive effects of combining the *rad3-102* and *rad27*-null alleles cannot be dismissed. Very similar effects were observed in diploids, suggesting that the presence of a homolog does not affect the mechanism of recombination. These results indicate that the *rad27*-null and *rad3-102* mutations may affect both

TABLE 3

Recombination and chromosome loss rates in wild-type and mutant strains

	Haploid		Diploid		
Relevant genotype	USCR[a] ($\times 10^{-6}$)	DRR[a] ($\times 10^{-5}$)	IHR[b] ($\times 10^{-5}$)	CL[b] ($\times 10^{-6}$)	DRR[c] ($\times 10^{-5}$)
Wild type	1 (0.8–1.2)	6.6 (5.5–7.8)	1.4 (1.2–1.5)	1.8 (1.4–2.5)	1.5 (1.2–2.7)
rad3-102	3.3 (1.3–5.6)	12 (11–13)	9.2 (8.3–11)	2.6 (1.4–4.2)	6.4 (4.9–18)
rad27	46 (26–61)	44 (32–59)	100 (90–120)	52 (33–67)	41 (33–56)
rad3-102 rad27	79 (54–107)	84 (32–220)	2000 (190–4000)	310 (149–612)	67 (44–120)

[a] USCR and direct-repeat recombination (DRR) rates were determined from a minimum of 15 trials as described in MATERIALS AND METHODS using haploids dissected from ABX761, ABX1308, ABX1368, and ABX1465; 95% confidence intervals are indicated in parentheses.

[b] IHR and chromosome loss (CL) rates were determined from a minimum of 35 trials as described in MATERIALS AND METHODS using diploids ABX633, ABX647, ABX658, and ABX693.

[c] DRR rates were determined from a minimum of seven trials as described in MATERIALS AND METHODS using diploids ABX1358, ABX1369, ABX1362, and ABX2010.

recombinagenic responses to replication lesions by similar mechanisms.

Together, the *rad3-102* and *rad27*-null mutations confer synergistic increases in chromosome loss and recombination between homologs: Chromosome loss and recombination between homologs have previously been shown to be stimulated in DNA replication mutant cells (HABER 1999), presumably in response to lesions generated during DNA replication. In particular, daughter-strand nicks or gaps that are not repaired by error-prone bypass or template switching with the sister chromatid may persist to form collapsed replication forks that can stimulate chromosome loss and recombination with the homolog in diploid cells (DAIGAKU *et al.* 2006). Since these may be important consequences of DNA replication lesion formation, we examined the epistatic interactions between *rad3-102* and the *rad27*-null allele with respect to chromosome loss and interhomolog recombination. Using a previously described assay for measuring the loss of one copy of chromosome V and recombination between chromosome V homologs (KLEIN 2001), we observed wild-type levels of chromosome loss and a sevenfold increase in interhomolog recombination in *rad3-102/rad3-102* homozygotes and a 29-fold increase in chromosome loss and a 71-fold increase in interhomolog recombination in the *rad27*-null/*rad27*-null homozygotes (Table 3). However, in contrast to the previous assays, the combination of the *rad3-102* and *rad27*-null alleles led to synergistically increased rates of chromosome loss and interhomolog recombination: a 172-fold increase in chromosome loss and a 1400-fold increase in interhomolog recombination in the *rad3-102/rad3-102 rad27*-null/*rad27*-null double homozygotes. These results suggest that DNA replication fork collapse is greatly stimulated in *rad3-102 rad27*-null mutants.

The *rad3-102* and *rad27*-null mutations together increase the percentage of G_2/M-phase cells with Rad52-YFP foci: Previously, LISBY *et al.* (2001, 2003, 2004) tagged the yeast Rad52 homologous recombination protein with the yellow fluorescent protein (YFP) (ORMÖ *et al.* 1996) and observed that it forms nuclear foci in response to DNA damage induced by ionizing radiation, DSB formation by the HO endonuclease, and collapsed replication forks. If, as suggested by the chromosome loss and interhomolog recombination data, the *rad3-102* and *rad27*-null alleles together lead to synergistic increases in replication fork collapse, more *rad3-102 rad27*-null double-mutant cells might be expected to display foci than *rad3-102* and *rad27*-null single mutant cells. As observed previously, we found that most wild-type cells exhibited diffuse nuclear fluorescence and that only 6.6% of S-phase and 4.1% of G_2/M-phase cells contained one or more foci in an asynchronous population (Table 4) (LISBY *et al.* 2001). Consistent with the levels of recombination observed in *rad3-102* and *rad27*-null mutant cells, 23% of S-phase and 14% of G_2/M-phase *rad3-102* mutant cells displayed a focus, while 23% of S-phase and 47% of G_2/M-phase *rad27*-null mutant cells displayed foci. As predicted, the *rad3-102 rad27*-null double mutant exhibited a greater percentage of cells with foci than either of the single mutants,

TABLE 4

Rad52-YFP focus formation in wild-type and mutant cells

	% cells containing a focus ±2 SE[a]	
Relevant genotype	S-phase	G_2/M-phase
Wild type	6.6 ± 5.3	4.1 ± 1.8
rad3-102	23 ± 3.8	14 ± 1.5
rad27	23 ± 7.6	47 ± 10
rad3-102 rad27	47 ± 8.0	68 ± 2.9

[a] Percentage of cells containing a focus was measured by dividing the number of cells in S- or G_2/M-phase containing one or more foci by the total number of cells in S- or G_2/M-phase and is reported as the median percentage ±2 standard errors of at least five trials. Each trial consisted of at least 50 cells.

TABLE 5

Doubling times of wild-type and mutant strains

Relevant genotype	Haploid DT ±2 SE (min)	Diploid DT ±2 SE (min)
Wild type	94 ± 3.9	95 ± 2.0
Wild type + pRS416	102 ± 2.7	ND
Wild type + pJM3	96 ± 3.4	ND
rad3-102	105 ± 3.9	98 ± 3.6
rad27	114 ± 8.0	130 ± 4.7
rad3-102 rad27	175 ± 11	130 ± 10
rad3-102 rad27 + pRS416	192 ± 11	ND
rad3-102 rad27 + pJM3	189 ± 11	ND

Doubling times (DT) were determined at 30° as described in MATERIALS AND METHODS and are reported as the median doubling time ±2 standard errors in minutes from at least five independent trials.

with 47% of S-phase and 68% of G_2/M phase cells containing foci.

A profound growth defect in the *rad3-102 rad27*-null double-mutant haploid is suppressed in the homozygous double-mutant diploid: The *rad3*-102 and *rad27*-null single mutant haploid strains exhibit mild growth defects at 30° (SOMMERS *et al.* 1995; SYMINGTON 1998) (Table 5). However, combining the *rad3-102* and *rad27*-null alleles synergistically increases doubling time in haploids. Strikingly, the doubling time of the *rad3-102*/*rad3-102 rad27*-null/*rad27*-null double homozygote is very similar to that in the *rad27*-null/*rad27*-null single homozygote (Table 5). This result may indicate an effect of diploidy, *MAT* heterozygosity, or both. *MAT* heterozygosity has been demonstrated to increase recombination in diploid cells as well as resistance to DNA damage in both haploid and diploid cells (FRIIS and ROMAN 1968; HEUDE and FABRE 1993; FASULLO and DAVE 1994; FASULLO *et al.* 1999). To distinguish whether *MAT* heterozygosity is solely responsible for rescuing the growth defect of the double-mutant cells, we transformed both wild-type and *rad3-102 rad27*-null *MAT*α haploids with a plasmid that contains the *MAT***a** sequence and repeated the growth assays. As a control, we also transformed wild-type and double-mutant *MAT*α haploids with pRS416 that lacks *MAT* sequences. *MAT* heterozygosity did not significantly alter the doubling time of *rad3-102 rad27*-null double-mutant haploids. This suggests that the presence of a homolog is required to rescue the growth defect imposed by the combination of the *rad3-102* and *rad27*-null alleles, perhaps by permitting chromosome loss or by enabling interhomolog recombination to rescue collapsed forks that otherwise inhibit growth.

DISCUSSION

The TFIIH and NER helicase Rad3 has been variously implicated in spontaneous mutagenesis and recombination, as well as in the processing of recombination intermediates (MALONE and HOEKSTRA 1984; MONTELONE *et al.* 1988, 1992; MONTELONE and LIANG-CHONG 1993; MONTELONE and MALONE 1994; BAILIS *et al.* 1995; MONTELONE and KOELLIKER 1995; BAILIS and MAINES 1996). This article addresses the effect of a known hypermutagenic and hyper-recombinagenic allele, *rad3-102*, in the context of the defined DNA replication defect conferred by the *rad27*-null mutation to better understand how Rad3 functions in the development of mutations, genome rearrangements, and LOH. We revealed that two mechanisms that promote LOH, chromosome loss, and interhomolog recombination were synergistically stimulated in the *rad3-102 rad27*-null double mutant. However, mutation rate and unequal sister-chromatid and direct-repeat recombination, mechanisms that do not rely on the presence of a homologous chromosome, were not stimulated any further in the double mutant than in the *rad27*-null single mutant. These results suggest that *rad3-102* confers a preference for mechanisms that utilize a homolog in the rescue of replication lesions generated in the absence of Rad27. In support of this hypothesis, differential growth of *rad3-102 rad27*-null haploids and homozygous diploids suggests that these lesions, which we suggest may be collapsed forks, either are efficiently repaired by interhomolog recombination or result in chromosome loss in diploids.

The Rad27 nuclease functions primarily in the removal of the 5′ RNA/DNA flap generated during lagging-strand synthesis (HARRINGTON and LIEBER 1994; REAGAN *et al.* 1995; SOMMERS *et al.* 1995; LIEBER 1997). In its absence, processing by other nucleases may create nicks or gaps in the daughter-strand, which have been observed in *rad27*-null mutants (VALLEN and CROSS 1995; PARENTEAU and WELLINGER 1999). These daughter-strand nicks and gaps may be utilized for mutagenic bypass or template switching and give rise to mutations or sister-chromatid exchange events (FASULLO and DAVIS 1987; HOLMES and HABER 1999; MICHEL *et al.* 2004; SALEH-GOHARI *et al.* 2005; LOPES *et al.* 2006b), as observed in our *rad27*-null mutant (Tables 2 and 3). Persistence of a flap would lead to replication fork collapse upon confrontation with the replicative polymerase during the next cell cycle. Chromosome breaks have been observed in *rad27*-null mutant cells (VALLEN and CROSS 1995; CALLAHAN *et al.* 2003), which could elicit checkpoint signals that lead to arrest in G_2, slow growth, and enhanced chromosome loss and interhomolog recombination (Tables 3 and 5; L. BI and A. M. BAILIS, unpublished results). These observations are most consistent with the *rad27*-null mutation primarily affecting the creation of DNA damage. Previous studies have implicated Rad27 in the processing of recombination intermediates (WU *et al.* 1999; NEGRITTO *et al.* 2001; KIKUCHI *et al.* 2005; ZHENG *et al.* 2005). The results reported here do not support such a conclusion, perhaps, because the effects of increased chromosome breakage on our assays eclipse the effects on the processing of

intermediates in *rad27*-null mutant cells. Substantial increases in all of the consequences of replication lesion formation in the *rad27*-null mutant cells argues that blocking Okazaki fragment maturation leads to dramatic increases in genome instability.

The role that the Rad3 helicase plays in the maintenance of genome stability is unclear. While the participation of Rad3 in NER and transcription is not inconsistent with the effects of *rad3* mutations on mutagenesis and recombination, *rad3-102*, the hypermutagenic and hyper-recombinagenic mutant described here, displays minimal or no defects in nucleotide excision repair or transcription (Hoekstra and Malone 1987; Montelone *et al.* 1988). Certain mutations in *SSL1*, which encodes another core subunit of TFIIH and the NER repairosome, stimulate short-sequence recombination and attenuate the processing of DSBs (Maines *et al.* 1998). Recently, Ssl1 has also been shown to have ubiquitin ligase activity that may influence its role in genome stability (Hoege *et al.* 2002). Since *RAD3* and *SSL1* have been shown to interact genetically (Bardwell *et al.* 1994; Maines *et al.* 1998), and Rad3 and Ssl1 to interact physically (Bardwell *et al.* 1994; Maines *et al.* 1998), it is possible that *rad3-102* influences genome stability through an interaction between Rad3 and Ssl1, but this has yet to be explored.

It was previously suggested that the hyper-recombinagenic effect of *rad3-102* resulted from the mutant protein generating lesions in the DNA that ultimately became DSBs (Montelone *et al.* 1988). This hypothesis was especially attractive since *rad3-102*, like *rad27*Δ, is synthetically lethal in mutant *rad52* and *rad50* backgrounds (Malone and Hoekstra 1984). While the hypermutagenic and hyper-recombinagenic nature of the *rad3-102* mutant is reminiscent of the *rad27*-null mutant, we observed several essential differences. In general, the hypermutagenic and hyper-recombinagenic characteristics of the *rad3-102* mutant were significantly less severe than those of the *rad27*-null mutant. These are reflected by the minimal effects of the *rad3-102* allele on cell-cycle profile, Rad52-YFP focus formation, and growth, which are inconsistent with substantial increases in DNA replication lesions (Tables 4 and 5; L. Bi and A. M. Bailis, unpublished results). Therefore, it seems likely that the *rad3-102* allele exerts its effect subsequent to the formation of DNA replication lesions. In further support of this hypothesis, we have previously shown that another allele of *RAD3*, *rad3-G595R*, has a substantial effect on recombination that has been initiated by an HO-endonuclease-catalyzed DSB, perhaps by changing their exonucleolytic processing (Bailis *et al.* 1995). Consequently, we suggest that the *rad3-102* allele may exacerbate the effect of the *rad27*-null allele on loss of heterozygosity primarily by altering the cellular response to DNA replication lesions.

The data presented here are consistent with a model for the interaction between the *rad27*-null and *rad3-102* mutant alleles where the loss of Rad27 leads to the accumulation of DNA replication lesions and *rad3-102* alters their processing. Loss of Rad27 results in the inefficient cleavage of RNA primer sequences from the 5′-ends of Okazaki fragments, such that at least one RNA residue remains, blocking their ligation to adjacent fragments. Other nucleases remove these 5′-ends, creating daughter-strand nicks and gaps that accumulate in *rad27*-null mutant cells (Merrill and Holm 1998; Parenteau and Wellinger 1999). The 3′-ends of these gaps may be recognized by polymerases with an increased tendency for mis-insertion that synthesize across the gap in an error-prone manner, contributing to the robust mutation rate in the *rad27*-null mutants (Table 2). Alternatively, the gaps may be repaired by template switching with the sister chromatid (Zheng and Fasullo 2003) that can also account for the duplications that accumulate in *rad27*-null mutants (Tishkoff *et al.* 1997), as well as for the increases in USCR and direct-repeat recombination (Table 3).

We suggest that *rad3-102* may attenuate the removal of the residual RNA primer sequences from Okazaki fragments by blocking nucleases that can compensate for the loss of Rad27 (Symington 1998). This may occur because the NER repairosome, of which Rad3 is a component, may recognize and bind to the primer sequences as it does to other polymerase-blocking lesions (Johansson *et al.* 2004), limiting nuclease access in *rad3-102* mutant cells. Under these circumstances, unprocessed and unligatable Okazaki fragments would be expected to accumulate. An increase in unprocessed Okazaki fragments may lead to widespread DSB formation by DNA replication fork collapse in the subsequent S-phase as the discontinuities would lie on the template for leading-strand synthesis (Figure 1). Alternatively, the nicks may stimulate DSB formation prior to the following S-phase (Tishkoff et *al.* 1997). The DSBs may be repaired by interhomolog recombination or give rise to chromosome loss that can be tolerated in diploids but may be fatal in haploids. Evidence for this in *rad3-102 rad27*-null double-mutant haploids exists in the significant increase in the percentage of cells with Rad52-YFP foci (Table 4) and the synthetic growth defect in haploid cells that lack an efficient means of rescuing the collapsed forks (Table 5). However, in *rad3-102 rad27*-null double-mutant diploids, the presence of a homolog appears to rescue the synthetic growth defect (Table 5), perhaps through the repair of DSBs by interhomolog recombination, which is increased 1400-fold, or through supporting chromosome loss, which is stimulated 172-fold (Table 3). Interestingly, the presence of a homolog failed to stimulate direct-repeat recombination in the *rad3-102 rad27*-null double-mutant diploids beyond that observed in the *rad27*-null single-mutant haploids, and these rates were not significantly different from those observed in *rad27*-null and *rad3-102 rad27*-null haploids (Table 3). This is consistent with fork collapse leading to the formation of single-ended DSBs that are thought to be ideal substrates

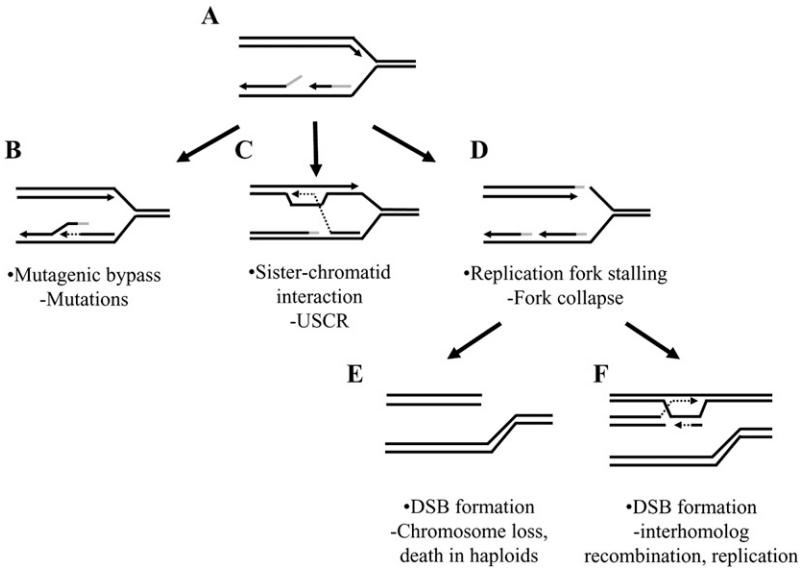

FIGURE 1.—Model of the consequences of DNA replication fork failure in *rad27*-null mutant cells. (A) During DNA replication, the 5′ RNA/DNA flaps (shaded line) of Okazaki fragments generated on the lagging strand are inefficiently processed in the *rad27*-null mutant. (B) The 5′ RNA/DNA flap may be displaced by mutagenic polymerases and later cleaved by other endo-/exonucleases. (C) Alternatively, the 3′-end of the next Okazaki fragment might interact with the sister chromatid to facilitate synthesis beyond the unligatable flap that is later displaced by the newly synthesized strand and degraded by exonucleases. (D) Unligated ends may persist until the next round of replication where they will serve as the leading-strand template and consequently force the fork to collapse, creating single-ended DSBs that may not be optimal substrates for template switching or direct-repeat recombination. This often results in chromosome loss and death in haploids (E), whereas in diploid cells (F), recombination with the homolog enables restart of the replication fork. Defective exonucleolytic digestion of residual flaps in *rad3-102 rad27*-null double mutants increases the incidence of unligated ends and results in synergistic increases in chromosome loss and interhomolog recombination in diploids, but kills haploids.

for interhomolog recombination by BIR (McEachern and Haber 2006), but not, perhaps, direct-repeat recombination, which is thought to occur by single-strand annealing (Lin *et al.* 1990; Ivanov *et al.* 1996; Dong and Fasullo 2003; Davis and Symington 2004).

The interhomolog recombination observed in wild-type diploids is largely *RAD51* independent (Klein 2001; Table 6), suggesting that the *rad3-102* allele may stimulate a *RAD51*-independent form of BIR. In support of this notion, *rad3-102* is not synthetically lethal in combination with the *rad51*-null allele (M. S. Navarro and A. M. Bailis, unpublished results). Interestingly, when we modified the interhomolog recombination assay such that gene conversion could be distinguished from BIR, we observed that a six- to sevenfold increase in gene conversion and BIR in a *rad3-102* diploid was suppressed in a *rad3-102 rad51*-null diploid (Table 6). This may suggest that the large stimulation in interhomolog recombination observed in the *rad3-102 rad27*-null diploids occurs by a Rad51-dependent mechanism. However, this hypothesis cannot be directly addressed due to the inviability of *rad27*-null *rad51*-null double-mutant cells (Tishkoff *et al.* 1997; Symington 1998; Debrauwere *et al.* 2001).

The high degree of conservation of the DNA replication and repair apparatus throughout eukaryotic phylogeny supports the speculation that similar genetic or pharmacological disruptions of Okazaki fragment maturation and processing in human cells could lead to massive increases in LOH, and the initiation of carcinogenesis. In fact, such collisions between pharmacology and genotype may help to explain differential responses to chemotherapeutic drugs, some of which disrupt DNA synthesis in a manner that may elicit unforeseen DNA repair responses. We are further exploring the role that Rad3, and, by extension, its human homolog Xpd, may play in the response to replication lesions at the DNA level in the hope of better understanding the link between DNA replication and LOH through homologous recombination.

TABLE 6

BIR and GC rates in wild-type and mutant diploids

Relevant genotype	BIR rate[a] ($\times 10^{-5}$)	GC rate[a] ($\times 10^{-5}$)
Wild type	1.3 (0.93–1.8)	0.22 (0.17–0.30)
rad3-102	8.5 (7.3–11)	1.3 (0.97–1.6)
rad51	3.5 (2.9–5.0)	0.37 (0.24–0.75)
rad3-102 rad51	4.6 (3.4–6.5)	0.47 (0.24–0.89)

[a] BIR and gene conversion (GC) rates were determined from a minimum of seven trials in the diploids ABX1498, ABX1204, ABX1175, and ABX1611; 95% confidence intervals are indicated in parentheses.

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