Efficient repair of large DNA loops in *Saccharomyces cerevisiae*

Stephanie E. Corrette-Bennett[1], Natasha L. Mohlman[1], Zulma Rosado[1], Juan José Miret[1,2], Patricia M. Hess[2], Breck O. Parker[2] and Robert S. Lahue[1,2,*]

[1]The Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Box 986805, Omaha, NE 68198-6805, USA and [2]Department of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, Worcester, MA 01655, USA

ABSTRACT

Small looped mispairs are efficiently corrected by mismatch repair. The situation with larger loops is less clear. Repair activity on large loops has been reported as anywhere from very low to quite efficient. There is also uncertainty about how many loop repair activities exist and whether any are conserved. To help address these issues, we studied large loop repair in *Saccharomyces cerevisiae* using *in vivo* and *in vitro* assays. Transformation of heteroduplexes containing 1, 16 or 38 nt loops led to >90% repair for all three substrates. Repair of the 38 base loop occurred independently of mutations in key genes for mismatch repair (MR) and nucleotide excision repair (NER), unlike other reported loop repair functions in yeast. Correction of the 16 base loop was mostly independent of MR, indicating that large loop repair predominates for this size heterology. Similarities between mammalian and yeast large loop repair were suggested by the inhibitory effects of loop secondary structure and by the role of defined nicks on the relative proportions of loop removal and loop retention products. These observations indicate a robust large loop repair pathway in yeast, distinct from MR and NER, and conserved in mammals.

INTRODUCTION

DNA mismatch repair (MR) is a powerful mutation avoidance pathway for base pair substitution and small insertion/deletion mutations (reviewed in 1–4). Highly conserved from bacteria to humans, MR acts on base–base mispairs and small loops, ranging up to 8–13 nt in eukaryotes (5–7). MR action eliminates these pre-mutagenic lesions before they can be fixed as mutations. Cells lacking MR exhibit mutation rates that are typically 100–1000 times higher than normal,

indicating that MR is a major activity for eliminating mispairs and small loops.

The correction of large looped mispairs presents a more complicated picture. Large loops are defined here as containing 15 nt or more and thus they are probably outside the realm of MR. In prokaryotes, there is little (8,9) or no detectable (10–12) processing of large loops. In yeast, some experiments also suggest poor processing of large loops (6,13–15). Other reports indicate three different loop repair activities in yeast that are distinguishable by their genetic requirements. One activity corrects loops of 26–30 nt during meiotic recombination and requires the MR gene *MSH2* and the nucleotide excision repair (NER) gene *RAD1* (16). A second pathway (17) functions on very large loops (>2 kb) in mitotic recombination, and activity is reduced by mutations in the MR genes *MSH2* or *PMS1*. A third activity, requiring *MSH3* and *RAD1*, prevents 92 bp deletions that result from replicational slippage errors (18,19). It has been suggested (15,20) that yeast loop processing activities might be of low capacity and thus easily saturable under conditions where excess loops are created, such as in certain replication mutant strains (15,20–24). Alternatively, some of these mutants could be deficient in loop repair activity.

There is also evidence of multiple loop repair pathways in mammalian cells. Transfection studies demonstrated efficient correction of heteroduplexes with loops up to 283 nt (25,26). Repair in this system can either remove the loop or retain it, in proportions of about 2:1 (25). Similar conclusions about the effectiveness of large loop repair (LLR) in mammalian cells have been drawn from recombination experiments (27–29), although the loops tested were smaller. Biochemical experiments using extracts of human cells also showed active repair of loops of 12–216 nt (5,30). Repair *in vitro* is strongly nick-dependent, with most repair occurring on the nicked strand, regardless of which strand contained the loop (30). This activity is independent of MR and NER genes. It is possible that the loop repair activities seen by transfection and *in vitro* are due to the same pathway. A third loop repair function was observed during mitotic recombination in rodent cells (31). Correction by this activity is unusual in that both palindromic

*To whom correspondence should be addressed at: The Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Box 986805, Omaha, NE 68198-6805, USA. Tel: +1 402 559 4619; Fax: +1 402 559 8270; Email: rlahue@unmc.edu
Present addresses:
Juan José Miret, OSI Pharmaceuticals Inc., 777 Old Sawmill River Road, Tarrytown, NY 10591-6705, USA
Breck O. Parker, Schleicher & Schuell, 10 Optical Avenue, PO Box 2012, Keene, NH 03431, USA

and non-palindromic sequences are efficiently repaired, suggesting that secondary structure associated with palindromic sequences can be overcome. In most other situations, secondary structure within the loop inhibits repair (27,32–35).

In contrast to other loop repair activities in yeast, we showed previously (36) that yeast nuclear extracts support an efficient LLR activity. This repair pathway functions on loops of 16–216 nt and there is full activity in extracts from *msh2 msh3* and *mlh1 pms1* mutant strains, although the role of the NER gene *RAD1* was not clearly determined. Our yeast LLR system appears to mirror activities seen in mammalian cell culture (25,26) and in extracts of human cells (30), judging by the high efficiency of repair, the loop sizes that are corrected and the independence of MR.

The biochemical results with yeast LLR encouraged us to examine this activity *in vivo*. We show in this study that LLR is very active in yeast, approaching 100% efficiency in transformation experiments. Tests with mutant strains demonstrated that LLR *in vivo* is independent of MR and NER genes, proving that LLR is distinct from the other yeast loop repair systems (16–19). We also found that secondary structure in the loop inhibits LLR both *in vivo* and *in vitro*, suggesting a difference from a palindrome processing activity found in rodent cells (31). In addition to the *in vivo* experiments, we report new biochemical experiments showing preferential nick-directed repair when the nick is located 5′ to the loop, compared to a 3′ nicked substrate. Together, these experiments provide new genetic and biochemical evidence for a robust, conserved large loop processing pathway among eukaryotes.

MATERIALS AND METHODS

Reagents and enzymes

Standard reagents, including molecular biology grade CsCl and low melting point agarose, were obtained from Sigma. Hydroxyapatite resin was a product of Bio-Rad. Vistra Green dye for DNA quantitation was from Amersham Life Sciences. All restriction enzymes were obtained from New England Biolabs or Stratagene. Exonuclease V was from US Biochemical Corp. Enzymatic reactions were performed as recommended by the manufacturers.

Heteroduplex construction

The large loop substrates used in the *in vivo* assays are derivatives of one parent plasmid, pHD5. pHD5 was created in our laboratory by cloning the *Hin*dIII–*Bam*HI fragment of the *ADE8* gene into the pRS313 vector (37). The resulting plasmid contains: (i) an origin of replication and the *bla* gene for propagation in *Escherichia coli*; (ii) an f1 origin which allows for the production of ssDNA by superinfection with a helper phage; (iii) the *HIS3* gene for selection of transformed yeast cells; and (iv) a centromere region and an ARS element for chromosome-like segregation in yeast cells. Expression of the *ADE8* gene is under the endogenous promoter. Derivatives of pHD5 were created in our laboratory by insertion of duplex oligomers at the *Hpa*I site, 135 bp from the ATG start codon, in the *ADE8* gene. In all cases, this is an out-of-frame disruption which produces an *ade8* mutation. The resulting plasmids contain the following inserted sequences and underlined diagnostic restriction sites: pHD16, 5′-AATT-

GCTAGCAAGCTT-3′, *Nhe*I; pHD38, 5′-CGACCCCTGTTG-CTGCCTCGAGGGCCGCGTCTTTGTCG-3′, *Xho*I; pCTG38, 5′-CGACCTGCTGCTGCTGGAATTCCTGCTGCTGCTG-GTCG-3′, *Eco*RI; pHPN38, 5′-CGACCTCGTCCTGCTCGC-ATGCGTGCTGGTCGTGGTCG-3′, *Sph*I. These sequences are on the viral strand of the phage, as confirmed by sequencing. DNA from these phage was used to create heteroduplex molecules as described below. A one base deletion at the middle nucleotide of codon 46, used to create a one base loop heteroduplex (14), was from W. Kramer (University of Göttingen).

Double-stranded DNA from the pHD5 vector and derivatives was purified from the *E.coli* strain JM101 [F′ *traD36 lacI*q Δ(*lacZ*)*M15 proA*+*B*+/*supE thi* Δ(*lac-proAB*)] using published procedures (38). Single-stranded DNA was produced in the *E.coli* X-90 strain [*ara* Δ*lac-pro nalA argEamb rifR thi1*–/F′ *lac*+ *pro*+ *lacI*(*Q1*)] by superinfection with the helper phage R408 (from K. Knight, University of Massachusetts Medical Center) at a multiplicity of infection of 10 and isolated using a CsCl purification method (38). Heteroduplexes for the transformation assay were prepared by the method of Lu *et al.* (39) with the following slight modifications. Double-stranded DNA harboring the desired sequence on the complementary (C) strand was linearized with *Bss*HII. The linear product was mixed with a 10-fold excess (w/w) of single-stranded circular DNA containing the viral (V) strand sequence. NaOH denaturation and subsequent neutralization resulted in heteroduplex formation. Isolation of the heteroduplex form required two consecutive purifications by electrophoresis on a 1% TPE (36 mM Tris base, 30 mM NaH$_2$PO$_4$, 1 mM EDTA) agarose gel. A final purification was performed by electrophoresis on a 1% TAE (40 mM Tris–acetate, 1 mM EDTA) low melting point agarose gel (Sigma). The DNA was subsequently released from the gel slice by treatment with β-agarase (New England Biolabs) according to the manufacturer's recommendations. Final heteroduplex preparations were ≥98% pure, as determined by agarose gel electrophoresis. Heteroduplexes were created by combining the respective C and V strands from the following pHD vector derivatives: V$_1$, pHD8 + pHD5; V$_{16}$, pHD5 + pHD16; V$_{38}$, pHD5 + pHD38; V$_{38}$ CTG, pHD5 + pCTG38; V$_{38}$ hairpin, pHD5 + pHPN38.

For *in vitro* assays, f1 phage MR9 and MR11 were kindly provided by P. Modrich (Duke University). Additional phage variants were created in our laboratory by insertion of duplex oligonucleotides. The resulting phage contain the following inserted sequences and underlined diagnostic restriction sites: MR9+30, 5′-CCCTGTTGCTGCCTCGAGGGCCGCG-TCTTT-3′, *Xho*I; MR9+CTG, 5′-CTGCTGCTGCTGGAAT-TCCTGCTGCTGCTG-3′, *Eco*RI; MR9+HPN, 5′-CTCGTC-CTGCTCGCATGCGTGCTGGTCGTG-3′, *Sph*I. These sequences are on the viral strand of the phage, as confirmed by sequencing. Both single-stranded and double-stranded DNA from the f1 phage were purified from JM101 cells using published procedures (38). Heteroduplexes were prepared by the method of Lu *et al.* (39) with slight modifications (38). To create the 3′ nicked substrate, MR9 double-stranded DNA was linearized with *Sau*96I and combined with a 5-fold excess (w/w) of MR11 circular single-stranded DNA. The C$_{27}$ heteroduplex was formed using the method described above and treated with DNA ligase. The covalently closed molecules were purified by electrophoresis on a 1% TAE low melting point agarose gel in

the presence of 1 µg/ml ethidium bromide. The DNA was subsequently released from the gel slice by treatment with β-agarase as described above. A site-specific nick 3′ to the loop mispair was introduced on the viral strand using gpII protein (40), kindly provided by J. Genschel and P. Modrich (Duke University). The resulting nicked substrate was again purified by electrophoresis and treatment with β-agarase. Final preparations were ≥98% pure, as judged by agarose gel electrophoresis. Heteroduplexes were created by combining the respective C and V strands from the following phage variants: C_{27}, MR9 + MR11; V_{27}, MR11 + MR9; V_{30}, MR9 + (MR9+30); V_{30} CTG, MR9 + (MR9+CTG); V_{30} hairpin, MR9 + (MR9+HPN).

Yeast strains

Yeast strains used for the MR-deficient and the loop secondary structure transformation assays were either MW3317-21A (MATα *trp1 ade8ΔKpn ura3-52 hom3-10 met13 met4 ade2 his3-Kpn*) or isogenic derivatives. MW3317-21A and MW3317-21A-*pms1Δ* were from S. Fogel (University of California at Berkeley). Gene disruptions to yield *msh2*::Tn*10LUK*, *msh3*::*TRP1*, *mlh1*::*URA3* and *rad1*::*URA3* derivatives were performed by a single round of disruption. Disruption plasmids were kindly provided by R. Kolodner (University of California at San Diego) for *MSH2*, M. Liskay (Oregon Health Sciences University) for *MLH1* and L. Prakash (University of Texas Health Sciences) for *RAD1*. The disruption plasmid for *MSH3* was created in our laboratory. All derivatives were confirmed by Southern blotting and appropriate genetic tests.

To study LLR in exonuclease-deficient backgrounds, W303-1a (MAT**a** *leu2-3 112 trp1-1 ura3-1 can1-100 ade2-1 his3-11,13*) and the isogenic derivatives *rad2*::*TRP1* (LSY381-3D) and *rad27*::*TRP1* (YBL3) were kindly provided by L. Symington (Columbia University). The above strains were modified to complement our assay system. An *ade8* derivative was created by a 326 bp deletion (*Cla*I–*Eco*47III) 54 nt from the start codon in the *ADE8* gene, resulting in the following strains: BL494 (wild-type), BL217 (*rad2*) and BL218 (*rad27*). For biochemical experiments, extracts were prepared from the yeast strain *DY6* (MAT**a** *ura3-52 leu2 trp1 prb1-1122 pep4-3 prc1-407*; from B. Jones, Carnegie Mellon University, via T. Hsieh, Duke University).

In vivo loop repair assay

Approximately 100 ng purified heteroduplex DNA was used to transform yeast cells by a modified lithium acetate electroporation procedure (41). This procedure yielded an average of $0.1–1 \times 10^5$ transformants/µg heteroduplex DNA, depending on the strain. A 25 ml culture of the strain to be transformed was grown overnight in YPD medium at 30°C to an OD_{600} of 3.0. The cells were washed once in sterile water and resuspended in 0.5 ml each of 10× LTE (1 M lithium acetate, 100 mM Tris base, 100 mM EDTA) and 1 M lithium acetate. The cells were incubated at 30°C with gentle shaking (50 r.p.m.) for 45 min, then 125 µl of 1 M dithiothreitol (DTT) was added and the cells incubated for an additional 15 min. Two consecutive washes with sterile water and one wash with 1 M sorbitol were necessary after the lithium acetate/DTT treatment. The cells were resuspended in 250 µl of 1 M sorbitol and were transformed immediately. The heteroduplex was incubated with

40 µl of competent cells for 5 min on ice, electroporated at 1.5 kV and resuspended in 1 ml of cold 1 M sorbitol. Approximately 5–10 µl of the transformation mixture was plated onto selective plates (SC his– limited adenine + 1 M sorbitol) and grown at 30°C for 5–6 days. The proportion of red, white and red/white sectored colonies was determined. After the original colony count was performed, the transformant colonies were replica plated to YPG plates to test for petites. Correction for petites did not change the overall percentage of red, white and sectored colonies.

Nuclear extract preparation

Nuclear extracts were prepared essentially as described by Wang *et al.* (42), with modifications as listed (36). Final extract preparations typically contained 5–6 mg/ml of protein, as measured by the method of Lowry *et al.* (43) after precipitation with trichloroacetic acid.

In vitro loop repair assays

All loop repair assays were performed as described in Corrette-Bennett *et al.* (36) unless otherwise noted. All restriction digests for C_{27} and V_{27} contained 7 U *Bsp*106I (Stratagene) to linearize the DNA. Repair that led to removal of the loop was evaluated by addition of 5 U *Eco*RI for substrates 3′ C_{27} and 5′ V_{27}. Repair in favor of the loop was evaluated with 2.5 U *Nhe*I. In the V_{30}, V_{30} CTG and V_{30} hairpin substrates, loop removal was assessed by restriction with 5 U *Pvu*II, whereas loop retention was evaluated with 6 U *Xho*I, 5 U *Eco*RI or 5 U *Sph*I, respectively. Following incubation at 37°C for 60 min, restriction analysis was performed on a 1% agarose gel. Unless otherwise noted, the gels were stained in a 1:10 000 dilution of Vistra Green dye (Amersham Life Sciences) and densitometric analysis of repair efficiencies was performed using a Molecular Dynamics Storm 860 PhosphorImager using the Blue Fluorescence/Chemiluminescence mode. Repair activity was measured as DNA present in the 3.1 + 3.3 kb bands divided by the total heteroduplex recovered and then converted to fmol loop repaired/mg yeast nuclear protein/h.

RESULTS

Experimental rationale

The major purpose of this study was to examine LLR in yeast in a way that paralleled our biochemical experiments and also studies done in several mammalian systems. An *in vivo* assay was chosen as the major focus because it is a sensitive and biologically relevant system. The genetic assay also facilitates the use of MR and NER mutant strains to help distinguish between different reported loop repair systems. We monitored the correction of defined, preformed heteroduplexes. These assays focus directly on repair of the loop and therefore differ from other genetic assays that rely on formation of the loop via recombination or replication slippage, prior to repair. The use of pre-existing looped substrates also allows direct comparison with our previous *in vitro* work and with several mammalian studies.

In vivo assay for loop repair

Loop repair was examined (14) by creating loop-containing DNA molecules with a defined size and location of the

A

B

Unstructured Loops

C

Structured Loops
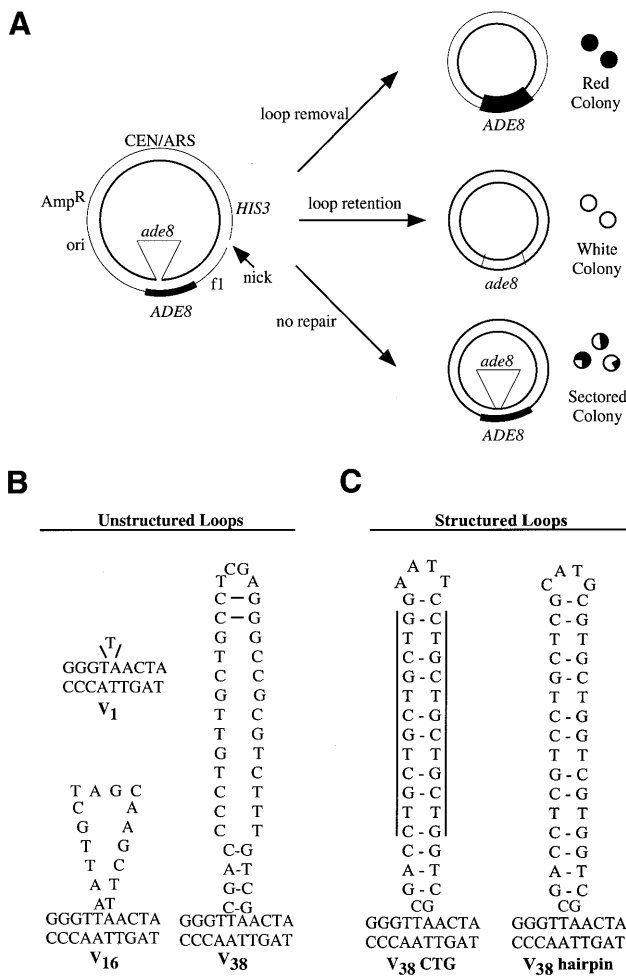
Figure 1. *In vivo* assay for LLR. The heteroduplex substrate, similar to that used by Kramer *et al.* (14), is diagrammed schematically in (**A**). The *ori* and Amp[R] genes are for propagation of the parental plasmids in *E.coli*. An f1 origin allows for the production of viral strand ssDNA by superinfection with a helper phage. The *HIS3* gene is used for selection of transformants. The *CEN/ARS* region provides an origin of replication and centromere function. In this example, a heteroduplex molecule was constructed in which the inserted sequence creates an *ade8* mutation in the inner, viral (V) strand, while the outer, complementary (C) strand of the substrate retains the wild-type *ADE8* sequence. A nick corresponding to a *Bss*HI site is placed 2.7 kb 3′ from the loop mispair on the complementary strand. The possible repair outcomes are described in the text. The looped heterologies used in this study are shown in (**B**). In the unstructured loops, the V indicates that the loop is located on the viral strand of the heteroduplex while the numeral indicates the number of nucleotides in the loop. The structured loops both have the potential to form secondary structures with 13 base pairings within the loop. The vertical lines for V_{38} CTG highlight the location of the CTG repeats.

heterology, followed by transformation into cells (Fig. 1A). The two strands of the substrate contain either a wild-type *ADE8* sequence or a frameshifted *ade8* allele. Placement of the loop in the *ADE8* gene allows for phenotypic monitoring of repair, as follows. The heteroduplex substrate is transformed into yeast *ade2 ade8* mutants and transformants are selected on synthetic complete medium lacking histidine, but containing limited adenine for color development. Correction of the

heteroduplex prior to replication results in all red or all white colonies. In this genetic background, the appearance of a red colony indicates repair that removes the loop (*ADE8*), whereas repair that retains the loop results in a white colony (*ade8*). If there is no repair, the colony shows a sectored red and white phenotype. The chromosomal *ade8* allele in this strain has a 1.3 kb *Kpn*I deletion that encompasses the site of the mispair for at least 250 bp on either side (14). Therefore, repair in these assays is unlikely to result from recombination between the heteroduplex substrate and the chromosome. It is possible that a sectored colony can arise by transformation of more than one heteroduplex molecule into a single cell. Repair to opposite strands in this case would result in a sectored phenotype. Co-transformation frequencies were estimated as the percentage of sectored colonies that appear when equal amounts of the plasmids pHD5 (*ADE8*) and pHD8 (*ade8*) were transformed into this strain. Co-transformation levels are estimated to be <5% based on these trials, similar to a previous report (14). Thus, co-transformation is negligible under these assay conditions.

Several different loops were used to examine LLR (Fig. 1B). We use a nomenclature in which V indicates that the loop is on the inner (viral) strand of the substrate. The numeral denotes the size of the loop in nucleotides. Thus, the V_{16} substrate contains a 16 nt loop on the inner strand (Fig. 1). The construction of the V_{16} and V_{38} substrates places the *ade8* gene on the looped strand, whereas a 1 nt deletion in the complementary strand of V_1 results in the viral strand harboring both the loop and the wild-type *ADE8* sequence (Fig. 1B). Previous *in vitro* work showed that efficient repair stimulated by a nick 114 bases 5′ of the loop mispair was reduced 3-fold when the nick was moved to 787 bases 5′ (36). This distance effect suggests that a nick engineered 2.7 kb 3′ of the heterology (Fig. 1A) is unlikely to initiate nick-stimulated repair in our transformation assays. Therefore, we used preformed heteroduplex substrates to directly address the role of the loop in signaling repair. V_{38} does have the potential to form a 4 bp hairpin at the base of the loop, but as shown by Petes and colleagues (33) and confirmed below, such limited structure-forming capacity does not impede correction.

Looped heteroduplex substrates are efficiently corrected *in vivo*

The V_{38}, V_{16} and V_1 unstructured loops were transformed into yeast cells and the resulting colonies were classified as red, white or sectored. Repair efficiency is determined by summing the number of red and white colonies and dividing by total colonies. In wild-type cells, repair of all three substrates is >90%, indicating that the loop is efficiently corrected (Fig. 2). Thus, preformed heteroduplex loops up to 38 bases are excellent repair substrates in yeast.

An advantage of this assay system is that we can determine repair efficiency to either strand, based on the red:white ratio. Both the V_{16} and V_{38} large loop substrates exhibit a correction bias, with a preference for loop removal over loop retention at a ratio of 2:1. In contrast, the substrate containing the 1 nt loop showed no strand preference, resulting in nearly equal numbers of red and white colonies, similar to the results of Kramer *et al.* (14). This difference in strand bias between the different size loops suggested that there is a unique mechanism directing repair of large loops.
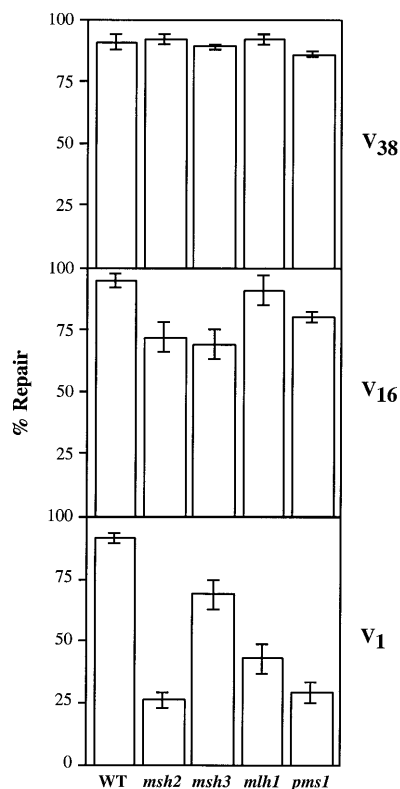
Figure 2. Loop repair activity in wild-type and MR-deficient strains. Repair activity was assayed on the V_1, V_{16} and V_{38} substrates as described in Materials and Methods. Repair of each heteroduplex was examined in the strain MW3317-21A (wild-type) and its isogenic derivatives containing null alleles of *msh2*, *msh3*, *mlh1* or *pms1*. Percent repair was determined as the sum of unsectored red and white colonies divided by total colonies. The error bars represent one standard deviation from at least three independent measurements.

Genetic independence of LLR from MR

In addition to assays in a wild-type background, the unstructured loop substrates were tested in isogenic mutant strains containing null alleles of the MR genes *msh2*, *msh3*, *mlh1* or *pms1*. V_{38} was designed as a substrate expected to be corrected by the LLR pathway, as determined previously by *in vitro* assays (30,36). The 16 nt loop size is near the border where MR and LLR may overlap and was used to determine the contributions of each of these pathways. The 1 nt loop was used as a control, as its repair efficiency is expected to decrease significantly when MR is inactivated.

The correction efficiency of the 38 nt loop in the MR-deficient strains remains essentially unchanged (85–95%) compared to wild-type, indicating that the loop is corrected by a pathway distinct from mismatch repair (Fig. 2). Since repair of the 38 nt substrate does not require *MSH2*, *MSH3* or *PMS1*, it differentiates LLR from other reported loop repair activities in yeast (16–19). The V_{16} substrate showed a small but significant decrease in repair in the *msh2*, *msh3* and *pms1* strain backgrounds. These data indicate that ~70–75% of total repair is by the LLR pathway, with the remaining 25–30% of repair

presumably due to MR. Loss of *mlh1* appears to have no effect on the correction of V_{16}. The 1 nt loop, as predicted, is affected by mutations in MR genes. The loss of *msh2* and *pms1* show the greatest deficiencies, with repair reduced to 26 and 29%, respectively. These results are consistent with those of Kramer *et al.* (14), where a *pms1* mutation reduced correction of a 1 nt loop to 19%, compared to a wild-type repair efficiency of 94%. In our experiment (Fig. 2), the *mlh1* strain exhibits a less severe repair defect, with correction reduced to 43%. In the *msh3* background, repair of the V_1 substrate is still 75% of wild-type. This limited dependence on *MSH3* was expected; the yeast Msh2p–Msh3p heterodimer primarily corrects loops 2–13 nt in size, playing only a small role in repair of 1 nt loops (44,45). This substrate specificity is reflected in our *in vivo* assay.

Genetic independence of loop repair from NER

In yeast meiotic recombination, LLR requires both *MSH2* and the nucleotide excision repair nuclease *RAD1* (16). Replicational slippage assays indicate that certain deletion events are inhibited by *MSH3* and *RAD1* (18,19). In our assays, *MSH2* and *MSH3* are clearly dispensable (Fig. 2; 36). To determine if components of the NER pathway are required for mitotic loop repair, we tested the V_{16} substrate in backgrounds deficient for the nucleases *rad1* and *rad2* (Fig. 3). Correction of the 16 nt loop is indistinguishable in two wild-type strains, suggesting that slight differences between the strain backgrounds do not affect LLR. Mutations in *rad1* or *rad2* do not show a significant effect on repair efficiency. These data suggest that repair of large loops is not dependent on these NER genes in mitotic cells.

A yeast strain harboring a *rad27Δ* mutation exhibits genomic instability which manifests as an increased number of large insertion and deletion mutations (21). This phenotype could be indicative of the loss of an LLR component. In addition, biochemical assays have indicated that LLR shows a preference for repair from a 5′ nick (30). The role of Rad27p as a 5′ nuclease, in combination with the mutator phenotye, led us to examine the effect of a *rad27Δ* mutation on LLR activity (Fig. 3). This mutant strain showed no loss of repair efficiency, suggesting that LLR is independent of *RAD27*. We cannot rule out the possibility that there are nucleases with redundant function that mask the effect of this single mutant, as seen for bacterial MR (46,47).

Secondary structure within loops reduces repair

In yeast recombination assays, loops predicted to form secondary structure result in an increased frequency of sectored colonies (32–35). This suggests that secondary structure within a loop inhibits repair. In these genetic assays, the formation of the loop is inferred, based on the final repair products. We utilized preformed heteroduplexes to examine directly the effect of loop structure on LLR efficiency. The sequences of our structured loop mispairs (Fig. 1B) were designed to mirror as closely as possible those used by Petes and colleagues (35), so that we could compare our mitotic results to those seen in meiosis. All three 38 nt loop substrates contain similar nucleotide compositions, but vary in sequence and base pairing capacity. The unstructured V_{38} substrate is used as a positive control and is repaired at ≥90% efficiency. The V_{38} CTG substrate contains a loop with the trinucleotide sequence CTG repeated eight times, resulting in 13 potential
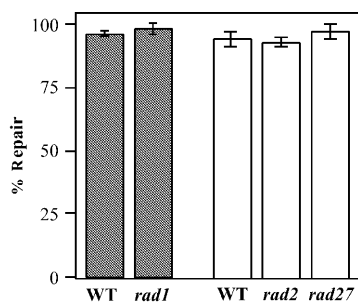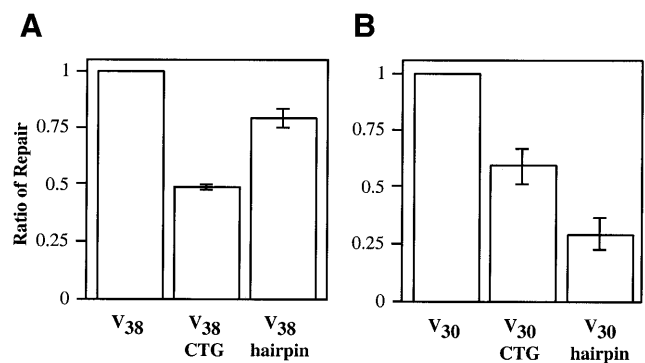
Figure 3. Loop repair activity in exonuclease-deficient strains. Repair activity *in vivo* was assayed on the V_{16} substrate as described in Materials and Methods. Analysis of repair for *rad1* was performed in the MW3317-21A strain background (shaded bars); *rad2* and *rad27* assays were in the BL494 background (white bars). Percent repair was determined as the sum of unsectored red and white colonies divided by total colonies. The error bars represent the standard deviation from at least three independent measurements.

Figure 4. Secondary structure within the loop affects repair. Repair of large loops was tested in both an *in vivo* transformation assay (**A**) and in a biochemical assay using nuclear extracts (**B**). The transformation assay is performed as described, using the V_{38}, V_{38} CTG and V_{38} hairpin heteroduplexes diagrammed schematically in Figure 1B. The ratio of repair is determined by normalizing total repair of the structured loops to that of the unstructured V_{38} loop. Total repair for V_{38} was 92%. A schematic representation of the biochemical assay is found in Figure 5A. The *in vitro* heteroduplexes used in this structural study are diagrammed schematically in Figure 5B. The ratio of repair, as described above, is normalized to the V_{30} substrate (an absolute value of 185 fmol/mg/h) in this biochemical assay. The error bars for both the *in vivo* and *in vitro* assays represent one standard deviation from three independent measurements.

base pairings within the loop. *In vivo* repair of the V_{38} CTG substrate is reduced by 50% when compared to the correction efficiency of the unstructured loop (Fig. 4A). Another way to view this result is that the percentage of sectored colonies, corresponding to unrepaired loops, increases 7-fold from 8% for the unstructured loop to 56% for the CTG repeat-containing heteroduplex. This decrease in correction efficiency could be due to secondary structure or to an intrinsic property of the repetitive primary sequence. To determine the potential contributions of structure and sequence, we designed another substrate, V_{38} hairpin, which contains the same number of G-C base pairings as V_{38} CTG, but in a non-repeating sequence (Fig. 1C). The V_{38} hairpin substrate reduced repair by ~25%. The ratio of loop removal to loop retention was reduced from the 2:1 value seen for the unstructured 38 nt loop to 1:1 for both the V_{38} CTG and V_{38} hairpin substrates. It appears that loop removal is more strongly inhibited by secondary structure than loop retention. From this information, we conclude that secondary structure within the loop does reduce LLR activity, however, the primary sequence of the loop may also contribute to the repair deficiency in our system.

Analysis of loop structure effects *in vitro*

We also tested the repair of structured loops *in vitro*, using an assay described previously (Fig. 5A) (36). Briefly, heteroduplex molecules were incubated with yeast nuclear extract, followed by restriction analysis to identify repair products. Three 30 nt substrates were designed to mimic those used in the *in vivo* assay (Fig. 5B). Whereas the V_{30} loop has little or no apparent secondary structure, the structured loops (V_{30} CTG and V_{30} hairpin) have the potential to form 9 bp within the loop. All three loop mispairs maintain similar nucleotide content. When normalized to the repair of the unstructured V_{30} loop, correction of the V_{30} CTG heteroduplex exhibits a 40% decrease in repair efficiency, similar to the results shown in the *in vivo* assay (Fig. 4B). In contrast, the V_{30} hairpin shows a 70% decrease in repair. To a first approximation, the inhibition of LLR by structure is similar *in vitro* to that seen *in vivo*. However, the relative inhibition by the two structured loops was different *in vivo*, where the V_{38} CTG loop was the more strongly inhibiting (Fig. 4A). One possible reason for the

difference between the transformation and the biochemical assay involves preparation of the nuclear extracts. Some of the protein components for correction of the hairpin substrate *in vivo* may not be efficiently released by our extraction protocol. A second possibility is that interactions of the loops with chromatin *in vivo* may not be recapitulated in the extract. Alternatively, there might be slight differences between the structures of the V_{30} hairpin and the V_{38} hairpin loop that affect inhibition of repair. A qualitative analysis suggests that both loop removal and loop retention were reduced *in vitro*, but the inhibited levels of loop retention with the structured substrates made it difficult to quantitate this effect. Taken together, the evidence from the *in vivo* and *in vitro* assays points to an inhibition of LLR by secondary structure within the loop.

Orientation of the nick affects nick-stimulated but not nick-independent repair

LLR in human cell extracts demonstrated that one of the distinct features of this repair pathway is a strong preference for a 5′ nick, compared to a 3′ break (30). In contrast, MR can utilize a 5′ or 3′ nick with similar efficiencies (5,48). We evaluated the utilization of 5′ and 3′ nicks in yeast extracts. A schematic of the heteroduplex substrates is found in Figure 6A. The two substrates are designed to be as similar as possible, with the orientation of the nick as the primary variable. Each heteroduplex molecule contains a 27 nt loop on one strand while the nick is placed on the opposing strand. Placing these two biologically important *cis*-elements on separate strands of the construct allows for monitoring of both possible outcomes of repair (36), because nick-stimulated repair directs excision to the outer, non-looped strand while nick-independent correction results in removal of the loop on the inner strand. The V_{27} molecule has a single nick 114 bases 5′ of the looped mispair, while a nick was introduced 170 bases 3′ of the heterology in the C_{27} molecule. Mapping of excision tracts in LLR indicates
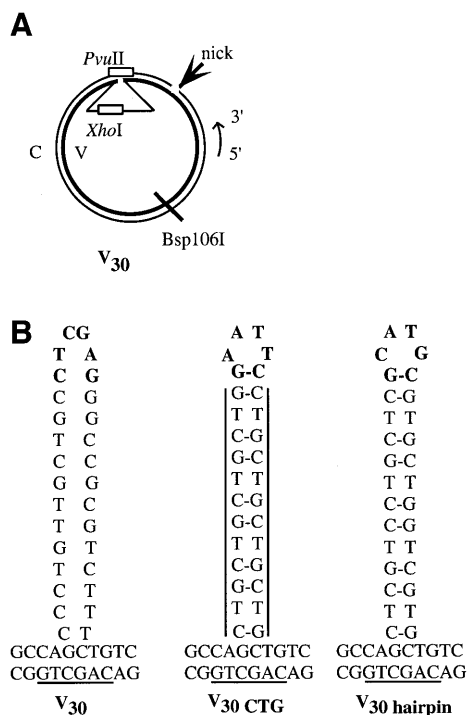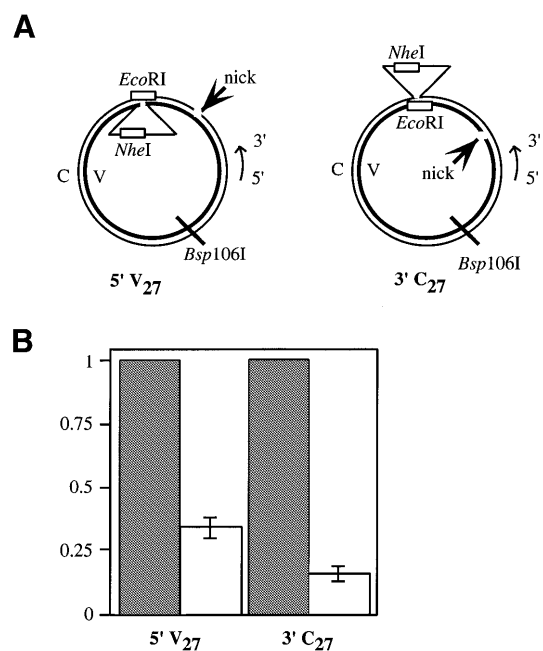
Figure 5. *In vitro* assay for LLR. The heteroduplex substrate with a loop on the viral (V) strand is diagrammed schematically in (**A**). Heteroduplex DNA molecules were constructed with a site-specific nick on the complementary (C) strand 114 bp 5′ to the loop. The V strand was covalently closed. The loop renders the heteroduplex resistant to both *Xho*I and *Pvu*II. If loop repair occurs, the DNA becomes sensitive to *Pvu*II if repair removes the loop, while the DNA is rendered sensitive to *Xho*I if the loop is retained. (**B**) The looped heterologies used in this study. A complete description of these molecules is included in the text.

Figure 6. Nick orientation affects LLR *in vitro*. The substrates used in this study are diagrammed schematically in (**A**). Each heteroduplex molecule contains a 27 nt loop on one strand while the nick is placed on the opposing strand. Nick-independent repair (loop removal) results in a molecule that is *Eco*RI^S. In contrast, nick-stimulated correction (loop retention) produces a *Nhe*I^S molecule. The V_{27} molecule has a single nick 114 bp 5′ of the looped mispair, while a nick was introduced 170 bp 3′ of the heterology by gpII protein in the C_{27} molecule. (**B**) A comparison of the correction efficiency of nick-stimulated repair (unfilled bars) relative to nick-independent repair (shaded bars). Absolute values for nick-independent correction activities are reported as fmol repaired heteroduplex/mg protein/h, and are as follows: 5′ V_{27}, 75 ± 2; 3′ C_{27}, 87 ± 2. The ratio of repair was determined by normalizing nick-stimulated repair to nick-independent correction. The error bars represent one standard deviation from three independent measurements.

that the proximity of these nicks to the loop mispair is close enough to activate nick-stimulated repair in our assay system (36). One minor difference between these two substrates is the primary sequence of the loop; V_{27} carries the viral strand sequence while C_{27} has the complementary strand sequence. Previous work indicates that, despite variations in the primary sequence, these loops have similar correction efficiencies (30,36).

After incubation in nuclear extracts, the DNA was tested for loop removal because this nick-independent reaction should be unaffected by nick orientation. Therefore, it is a convenient internal control. Absolute values for nick-independent loop removal (in fmol repaired heteroduplex/mg protein/h) were 75 ± 2 for 5′ V_{27} and 87 ± 2 for 3′ C_{27}. The comparable levels of loop removal in the two substrates supports the prediction of a nick-independent reaction. In contrast, the nick-stimulated repair showed a 2-fold preference for a 5′ nick. In Figure 6B, nick-stimulated repair of 5′ V_{27} was 30% as effective as loop removal, whereas nick-stimulated correction of the 3′ C_{27} heteroduplex is only 15% as effective as loop removal. This result indicates that while a 3′ nick may be utilized for excision, nick-stimulated LLR is more effective from a 5′ break.

DISCUSSION

One of the major findings of this study is that *in vivo* repair of large loops in *S.cerevisiae* can be very efficient. Transformation

assays with loops of 1, 16 or 38 nt showed high level, approximately equal correction levels for all three substrates. Under these assay conditions, LLR on the 38 nt loop was about as active as MR was on the 1 nt heterology. This extent of correction indicates that yeast has substantial capacity to correct loop sizes well beyond the range of MR. The second major conclusion from this study is that *in vivo* LLR in yeast shows close similarity to loop repair activities catalyzed by yeast extracts (36) and those seen in several mammalian systems (5,25,26,30,49). Taken together, these reports indicate a robust repair capacity on loops ranging from 16 to 283 nt. The similarities of LLR in yeast and mammalian cells suggests conservation of function in eukaryotes that is independent of numerous MR or NER components (30,36; this work). The identification of LLR protein components will be an important goal in the field.

LLR can be differentiated genetically from other loop repair activities reported in the yeast literature. For example, our assays show that mutation of either the NER nuclease encoded by *RAD1* or the MR *MSH2* gene product does not detectably alter repair on a 16 base target. These results indicate that loop repair in mitotic yeast cells occurs by a different pathway than in meiotic recombination (16). Similarly, the fact that LLR

activity does not require *MSH3* or *RAD1* distinguishes it from another loop correction activity that helps overcome certain replication slippage events (18,19). A third alternative pathway for very large loops (≥2 kb) formed during mitotic recombination is at least partially dependent on *MSH2* and *PMS1* (17). Clearly, the efficient LLR activity observed both *in vitro* (36) and *in vivo* (this study) occurs by a unique pathway in yeast. One possible explanation for the presence of multiple loop correction pathways in yeast may involve the manner in which the loops are formed. It has been suggested that accessibility to the loop might be modulated by other proteins, thus controlling which repair pathway can act to correct the loop (17,50). For example, a loop formed during recombination could conceivably be accessed by a different subset of repair proteins than a loop arising from replicational slippage. Our results indicate that, given a preformed looped heteroduplex, the yeast cell is capable of efficiently correcting heterologies up to 216 nt.

What features determine whether the loop is removed or retained? Like our transformation experiments in yeast, transfection experiments with mammalian cells showed that LLR has a preference for removal of the heterology (25,26,49), suggesting that the loop is the predominant *cis*-factor. The influence of a nick 71 or 125 bases from the loop was also tested in mammalian cells. The effect of the nick appeared to be small, regardless of its orientation (49). However, it could not be ascertained whether the nick persisted after transfection or was ligated. Biochemical experiments clearly indicate that a 5′ nick can be utilized to direct repair to the discontinuous strand. This preference is strong in human cell extracts (30) and also occurs to a lesser extent in yeast extracts (this study). One explanation for the reduced 5′ nick preference in yeast is that endogenous nicking activities in the extract might occasionally cleave the strand that already has the existing nick. If so, some of the apparent nick-stimulated repair could arise from secondary nicking activities, rather than utilization of the existing DNA terminus. This scenario would reduce the apparent 5′ versus 3′ nick preference observed in Figure 6, because some baseline level of nick-stimulated repair on both substrates would be catalyzed by endogenous nicking activity. This line of reasoning also suggests that nick-stimulated and nick-independent repair may not be completely distinct, if one accepts that single-strand cleavage on the continuous strand (leading to loop removal in Fig. 6) might also sometimes occur on the discontinuous strand. To address possible nucleases involved in either creating a nick or utilizing a pre-existing 5′ nick, we tested loop repair efficiency in single mutants of *RAD1*, *RAD2* and *RAD27*. However, repair of a 16 nt loop was at wild-type levels in these mutant strains, despite the fact that our *in vivo* assay is suitably sensitive for detection of minor defects in loop repair. Our results suggest that either these genes play no role in LLR or else there is a redundant counterpart.

The results reported here provide two insights into the inhibitory effect of secondary structure within the 38 nt loop. First, reduced loop repair activity on hairpins is distinct from an activity in human cells that can repair palindromic hairpins up to 40 bases in length (31). Secondly, our inhibition studies indicate that LLR is one of the DNA repair pathways that is overcome during expansion of trinucleotide repeats (51). The hairpins we tested, inspired by the work of Moore *et al.* (35), were designed to mimic replication intermediates thought to be important during the triplet repeat expansion process. The reduced repair of hairpin loops containing only eight CTG repeats suggests that LLR is unable to process mutagenic intermediates in important disease genes. Among trinucleotide repeat expansions in affected human families, an increase of eight repeats is relatively small. Many disease-causing expansions are significantly larger, in some cases increasing the tract by thousands of repeats (52). The fact that an eight CTG repeat loop inhibits LLR by 40–50% suggests that loops with more repeats will be even more inhibitory. Our findings help explain how mutagenic intermediates containing trinucleotide repeat sequences avoid LLR.

Gene conversion studies indicate that most insertions and deletions up to ~1 kb undergo frequent correction in yeast meiosis (53), suggesting the active repair of large loops. One exception is the *ade8-18* allele, a 38 base deletion that shows low gene conversion and high post-meiotic segregation (13). Heteroduplex loops created by annealing wild-type *ADE8* to *ade8-18* are also refractory to repair upon transformation (14). We suggest that resistance of the *ade8-18* allele to loop repair is the exception, rather than the rule, because LLR has been demonstrated for numerous other heterologies (16,25,26,30,35,36,53), including a different 38 nt loop (this study).

Our work suggests the presence of an active LLR activity in yeast. Yet experiments that examined the mutational spectrum created by certain mutant alleles of *POL3* (polymerase δ), *POL30* (PCNA) and *RAD27* (FEN-1) (15,20–24) show numerous insertion/deletion events of a size that should be prevented by this pathway. One possibility is that the number of strand slippage errors is so great in these mutants as to saturate LLR capacity. Another explanation, outlined earlier, is that accessibility of repair components is somehow modulated by other proteins involved in formation of the loop. A third possibility is that polymerase δ and PCNA may be involved in LLR. Thus it is possible that these mutations may also inhibit efficient correction of the heterologies.

Together, MR and LLR provide complementary correction of loops that range from one to hundreds of nucleotides. There appears to be overlap between the pathways for intermediate size loops. We found that repair of a 16 nt loop showed a partial (25–30%) dependence on *MSH2*, *MSH3* and *PMS1*. These results suggest that MR has, under certain circumstances, some activity on loops as large as 16 bases. In this respect, we were surprised to see that loss of *MLH1* did not affect repair of the 16 nt loop, although previous studies indicate that repair of a different 16 nt heterology occurs readily in extracts from human MLH1$^{-/-}$ cells (5). It seems unlikely that the disrupted *mlh1* allele we tested had residual activity, since repair of a 1 nt loop was significantly compromised. A more detailed analysis of the repair of intermediate size loops will be required to resolve these issues. What is clear from these studies is that overlapping substrate specificity of MR and LLR ensures coverage of a wide spectrum of possible loop sizes.

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