Opposing roles for DNA structure-specific proteins Rad1, Msh2, Msh3, and Sgs1 in yeast gene targeting

Lance D Langston¹ and Lorraine S Symington^{2,*}

¹Integrated Program in Cellular, Molecular, and Biophysical Studies, Columbia University Medical Center, New York, NY, USA and

²Department of Microbiology and Institute of Cancer Research, Columbia University Medical Center, New York, NY, USA

Targeted gene replacement (TGR) in yeast and mammalian cells is initiated by the two free ends of the linear targeting molecule, which invade their respective homologous sequences in the chromosome, leading to replacement of the targeted locus with a selectable gene from the targeting DNA. To study the postinvasion steps in recombination, we examined the effects of DNA structure-specific proteins on TGR frequency and heteroduplex DNA formation. In strains deleted of *RAD1*, *MSH2*, or *MSH3*, we find that the frequency of TGR is reduced and the mechanism of TGR is altered while the reverse is true for deletion of *SGS1*, suggesting that Rad1 and Msh2:Msh3 facilitate TGR while Sgs1 opposes it. The altered mechanism of TGR in the absence of Msh2:Msh3 and Rad1 reveals a separate role for these proteins in suppressing an alternate gene replacement pathway in which incorporation of both homology regions from a single strand of targeting DNA into heteroduplex with the targeted locus creates a mismatch between the selectable gene on the targeting DNA and the targeted gene in the chromosome.

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Introduction

Targeted gene replacement (TGR) has been a fundamental technique in molecular biology for more than 20 years, but little is known about the mechanism by which a targeted sequence in the genome is replaced with a selectable gene from the linear targeting DNA. Without flanking homology sequences to direct it, the marker integrates randomly into the genome, so TGR is clearly homology-driven, but its relationship to other forms of homologous recombination (HR) is unclear. TGR occurs with high efficiency in *Saccharomyces cerevisiae*, even with very short flanking homologies, but in mammalian cells the efficiency is much

lower. Because of the importance of this technique for genome manipulation and its potential use in gene therapy, understanding the mechanisms and limiting steps may result in new methods to improve the efficiency of TGR in mammalian cells.

Repair of DNA double-strand breaks (DSBs) by HR is a high-fidelity process that utilizes an undamaged homologous template to restore missing sequences at the break (Symington, 2002). By contrast, TGR can be considered a corruption of the HR process because recombination leads to permanent loss of the targeted chromosomal region (Rothstein, 1983) rather than restoration of a damaged sequence. In this respect, TGR is similar to another mutagenic HR process, the single-strand annealing (SSA) pathway of recombination between direct repeats (Lin *et al*, 1984; Ozenberger and Roeder, 1991; Fishman-Lobell *et al*, 1992). TGR and SSA both involve recombination using interrupted homology sequences and thus share unique genetic dependencies not found in other HR processes. Both pathways require the Msh2:Msh3 mismatch repair complex (Saparbaev *et al*, 1996) and the heterodimeric Rad1:Rad10 endonuclease (Schiestl and Prakash, 1988, 1990) to process intermediates leading to efficient recombination, which is generally not the case for template-directed repair of DSBs. Msh2:Msh3 and Rad1:Rad10 are also involved in repair of large insertion/deletion (loop) mismatches that can form between heteroalleles during meiotic recombination (Kirkpatrick and Petes, 1997; Kearney *et al*, 2001). Additionally, Msh2 plays a role in mitotic large loop repair (Clikeman *et al*, 2001), but the remaining factors have yet to be tested in the same assay system. Msh6, a mismatch repair protein specific for base–base and small insertion/deletion mispairs, is not required for SSA (Sugawara *et al*, 1997) or meiotic large-loop repair (Kearney *et al*, 2001), further supporting the idea that the role of mismatch repair factors in SSA is specific for branched DNA structures.

In yeast, SSA, TGR, and template-directed repair of DSBs are all strongly dependent on *RAD52* (Symington, 2002), which encodes the founding member of a superfamily of single-strand DNA (ssDNA) binding proteins (Iyer *et al*, 2002). TGR is most efficient when at least one of the 3' ssDNA ends of the targeting fragment invades a homologous chromosomal duplex in a reaction mediated by the Rad51 strand exchange protein and its accessory factors Rad55/57 (Schiestl *et al*, 1994) and Rad54 (Schmuckli-Maurer *et al*, 2003), but unlike Rad52, none of these proteins is absolutely required for TGR in yeast.

It has been firmly established both in cultured mammalian cells and in yeast that TGR is initiated by separate strand invasions at the two homology ends of the linear targeting molecule accompanied by formation of extensive heteroduplex DNA (hDNA) with the targeted chromosomal locus (Li *et al*, 2001; Langston and Symington, 2004). The steps that

*Corresponding author. Department of Microbiology and Institute of Cancer Research, Columbia University Medical Center, 701 W 168th Street, New York, NY 10032, USA. Tel.: +1 212 305 4793; Fax: +1 212 305 1741; E-mail: lss5@columbia.edu

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follow strand invasion are less clear, but branched DNA intermediates are likely to be involved because mutant cell lines deficient for proteins that act on these structures show altered frequencies of TGR. A role for RecQ helicases in suppressing TGR has been established in human cells and cultured chicken B cells, both of which have multiple RecQ homologs including three—*BLM*, *WRN*, and *RECQL4*—that are associated with human disease when mutated (Ellis *et al*, 1995; Yu *et al*, 1996; Kitao *et al*, 1999). Inactivation of *BLM* in a human colorectal cancer cell line increased the frequency of TGR approximately 20-fold at two different loci (Traverso *et al*, 2003), while disruption of *BLM* in the chicken DT40 cell line increased the frequency of TGR three- to 10-fold (Wang *et al*, 2000; Imamura *et al*, 2002). The human Blm protein binds to and migrates synthetic branched recombination intermediates *in vitro*, including Holliday junctions (Karow *et al*, 2000) and D-loops (van Brabant *et al*, 2000), so it might suppress TGR by unwinding similar intermediates.

The involvement of the structure-specific endonuclease Rad1:Rad10 suggests the formation of branched intermediates during TGR analogous to those found in SSA. The requirement for the mammalian Rad10 homolog, encoded by *ERCC1*, in TGR is absolute (Niedernhofer *et al*, 2001), while the requirement for Rad1:Rad10 in yeast is highly variable, depending on the targeted locus and perhaps on the targeting DNA itself. Separate studies have reported TGR defects in yeast *rad1* and *rad10* mutants ranging from a three- to four-fold reduction (Schiestl *et al*, 1994; Symington *et al*, 2000) to a 40-fold reduction (Schiestl and Prakash, 1988, 1990; Saparbaev *et al*, 1996). The less stringent requirement for Rad1:Rad10, along with the overall higher efficiency of gene targeting in yeast suggests the possibility of a fundamental difference between yeast and mammalian cells in the mechanism of TGR. To investigate this possibility, and to study the role of DNA structure-specific factors in the post-invasion steps of TGR in more detail, we looked for changes in gene targeting frequency and hDNA formation during TGR in strains of *S. cerevisiae* deleted of the *SGS1*, *RAD1*, *MSH2*, *MSH3*, and *MSH6* genes.

Results

Experimental system

As previously described, we used restriction site polymorphisms in the two homology regions flanking a selectable marker to monitor formation of heteroduplex between corresponding regions of the targeting DNA and the targeted chromosomal locus during TGR (Langston and Symington, 2004) (Figure 1). The restriction enzyme recognition sites were incorporated into 29 bp palindromic inserts that avoid mismatch repair by forming stable, internally complementary loops when paired with DNA lacking the insertion (Nag *et al*, 1989). Genomic DNA was prepared from isolated single colonies identified as transformants resulting from TGR. Each flanking homology region was analyzed by RFLP analysis after PCR amplification to determine if it contained the restriction enzyme recognition site from the targeting DNA, the chromosomal DNA, or both, the latter indicating a sector colony resulting from unrepaired hDNA (see Materials and methods for details). Transformants that exhibited hDNA in both flanking homology regions were analyzed further to determine if the same (*cis*; Figure 2, right) or

Figure 1 Schematics of targeting DNA and targeted yeast strains. Open arrows show the selectable markers, *TRP1* or *MET17*, and rectangles show the corresponding disrupted allele in the chromosome. (A) Targeting at the *TRP1* locus. (B) Targeting at the *MET17* locus of a complete disruption or (C) a point mutation. Restriction site polymorphisms in the flanking homology regions are indicated. This figure is adapted from Figure 2 of Langston and Symington (2004).

different (*trans*; Figure 2, left) strands of targeting DNA at each end interacted with the chromosomal DNA.

We previously examined hDNA formation in wild-type cells during targeted replacement of a chromosomal *trp1::URA3* site by *TRP1* (Figure 1A) and a chromosomal *met17::ADE2* site (where *ADE2* is under control of the *MET17* promoter) with *MET17* (Figure 1B). Of the 44 products of TGR that had hDNA in both flanking homology regions, 43 were in the *trans* configuration (diagrammed in Figure 2A), consistent with a model in which a different strand of targeting DNA at each end invades the chromosome as the dominant mechanism for TGR initiation in wild-type cells (Langston and Symington, 2004).

The mechanism and frequency of TGR are conspicuously altered in the absence of the *MSH2* and *RAD1* genes

One of the assumptions underlying our experimental system is that palindromic sequence polymorphisms in the flanking homology regions of the targeting DNA escape mismatch repair when hDNA is formed with the native chromosomal DNA. To confirm the validity of this assumption, we analyzed hDNA formation during TGR in strains deleted of *MSH2*, a homolog of *Escherichia coli* MutS and a key component of the yeast mismatch repair machinery (Reenan and Kolodner, 1992). Consistent with previous studies (Saparbaev *et al*, 1996), the frequency of TGR was reduced at both *MET17* (decreased four-fold, $P=0.01$; Figure 3A) and *TRP1* (decreased three-fold, $P=0.004$; Figure 3B) in the *msh2* strain. If hDNA in the flanking homology regions was undergoing mismatch repair, we would have expected to see an increased frequency of hDNA in *msh2* mutants. To our surprise, we found that the proportion of hDNA was reduced in the *msh2*

Figure 2 Models of targeted gene replacement. The large black box represents the selectable marker on the targeting DNA; small open boxes and open circles indicate different relative positions of palindromic restriction site inserts in the flanking homology regions of the *TRP1* and *MET17* targeting DNA, respectively; dotted lines indicate possible nuclease resection of the 5' ends of the targeting DNA. Uninterrupted lines indicate the targeted chromosomal locus. Bisected ovals at bottom show the different genotypes of the two daughter cells arising from TGR as shown. As indicated by the thick versus dotted lines, only daughter cells containing the targeting marker survive selection to form all or part of a colony. (A) TGR with flanking markers in *trans*. (B) No TGR. The large bubble mismatch between the selectable marker on the targeting DNA and the targeted chromosomal locus is unwound, or is repaired in favor of chromosomal sequences. (C) TGR with flanking markers in *cis*. The bubble mismatch is repaired in favor of the targeting DNA. (D) TGR with no hDNA observed. The bubble mismatch is not repaired, and all information from the top, chromosomal strand is lost under selection for the targeting DNA marker on the bottom strand.

Figure 3 Frequency of TGR. Number of TGR events per 100 ng of targeting DNA compared to the number of transformants per 1 ng of uncut control plasmid DNA. (A) Targeting of *met17::ADE2* in the indicated genetic background. (B) Targeting of *trp1::URA3*. Shown are the means and standard deviations of at least three experiments except for *rad1 mus81*, which is from two experiments.

mutant at both *MET17* (44/95, or 46% in *msh2* versus 65/94, or 69% for the isogenic *MSH2* strain, $P=0.002$; Figure 4A) and *TRP1* (43/94, or 46% in *msh2* versus 70/103, or 68% for *MSH2*, $P=0.002$; Figure 4B). More importantly, we observed that hDNA was altered significantly in the configuration of flanking markers, with eight out of 12 transformants (six of seven at *TRP1* and two of five at *MET17*) with the *cis* configuration in *msh2* versus one *cis* out of a total of 18 in the *MSH2* controls ($P=0.0006$; Figure 4, bottom). The reduced frequency of TGR and the considerable reduction in the number of transformants with hDNA in the *trans* configura-

tion in the absence of Msh2 suggests that this protein is important for facilitating the type of TGR found in wild-type cells. The shift to the *cis* configuration of flanking markers suggests that Msh2 also plays a role in restricting an alternate mechanism of TGR.

The structure-specific endonuclease encoded by the *ERCC1* and *XPF* genes is indispensable for TGR in mammalian cells (Niedernhofer *et al*, 2001). To investigate the role of the corresponding endonuclease in yeast, we examined TGR in strains deleted of *RAD1*, the homolog of *XPF*. In accordance with previously published results (Schiestl *et al*, 1994;

Figure 4 hDNA formation during TGR. (Top) For each strain, the percentage of colonies analyzed that had both vector and chromosomal restriction sites at one or both flanking markers is indicated by the dotted bars, those with vector restriction sites only at one or both flanking markers by open bars, and those with only chromosomal restriction sites at both flanking markers by gray bars. The number of colonies analyzed for each strain is indicated below the stacked bars. (Bottom) For each strain, the number of colonies that had both vector and chromosomal restriction sites at both flanking markers is indicated. Subclone analysis determined how many of these were in the *trans* or *cis* configurations. (A) Targeting of *met17::ADE2* in the indicated genetic background. (B) Targeting of *trp1::URA3*. Data for wild type in both (A) and (B) are adapted from Langston and Symington (2004).

Symington *et al.*, 2000), we observed a modest decline in TGR frequency in *rad1* mutants, reduced three-fold at *TRP1* ($P=0.001$; Figure 3B) and two-fold at *MET17* ($P=0.06$; Figure 3A) compared to wild-type controls. However, the proportion of transformants with hDNA was radically reduced in *rad1* mutants, with only 6/93 (6%) at *TRP1* and 15/94 (16%) at *MET17* showing hDNA formation in either flanking homology region compared to 70/103 (68%, $P<0.0001$) and 65/94 (69%, $P<0.0001$) in the corresponding *RAD1* strains (Figure 4). In *rad1* strains, only four transformants, all at *MET17*, formed hDNA at both ends, and all four of these were in the *trans* configuration (Figure 4A, bottom), suggesting that a small portion of two-end invasion intermediates can still be processed in the absence of Rad1. This alternative processing does not appear to be mediated by the Rad1-related protein Mus81, as the frequency of TGR in a strain mutated for both *MUS81* and *RAD1* was reduced only slightly compared to the *rad1* single mutant (Figure 3A).

The role of Msh2 in TGR is mediated by its interaction with Msh3 but not Msh6

Msh2 forms separate complexes with Msh3 and Msh6 to process different types of intermediates in mismatch repair and HR (Johnson *et al.*, 1996; Marsischky *et al.*, 1996; Saparbaev *et al.*, 1996). To determine if either of these proteins is involved in gene targeting, we analyzed TGR frequency and hDNA formation at *MET17* in *msh3* and *msh6* strains. As expected, deletion of *MSH6*, which is specific for base-base or very small insertion-deletion mispairs, had no effect on either the frequency (Figure 3A) or the mechanism (Figure 4A) of TGR. By contrast, deletion of *MSH3*, which is specific for larger insertion/deletion mis-

pairs, reduced the frequency of TGR two-fold when compared with either wild type ($P=0.12$) or *msh6* ($P=0.03$; Figure 3A) and altered the mechanics of hDNA formation in a manner similar to that of *msh2*, with 32/95 (34%) transformants showing hDNA in one or both flanking homology regions compared to 44/95 (46%, $P=0.10$) in *msh2*, 60/91 (66%, $P<0.0001$) in *msh6*, and 65/94 (69%, $P<0.0001$) in the wild-type *MSH3* strain (Figure 4A). Analysis of transformants with hDNA at both flanking markers showed 10/12 in the *trans* configuration in the *msh6* mutant while 4/4 were in the *cis* configuration in the *msh3* mutant ($P=0.008$, Figure 4A, bottom), confirming that the role of Msh2 in TGR is mediated by its interaction with Msh3, not Msh6.

Msh2:Msh3 and Rad1 operate together to prevent single-strand assimilation

Because the hDNA patterns observed in the *msh2* and *msh3* mutants were considerably different from those in *rad1*, we sought to determine whether Msh2:Msh3 and Rad1 affect the mechanism of TGR by the same or different pathways. To do so, we repeated the TGR experiments at *MET17* in an *msh2 rad1* double mutant. Differences in the frequency of TGR between the double mutant and the respective single mutants were not significant, but hDNA formation in the *msh2 rad1* strain closely resembled that of the *rad1* single mutant and was significantly different from the *msh2* single mutant. In all, 7/96 (7%) transformants showed hDNA in one or both flanking homology regions in the *msh2 rad1* double mutant compared to 15/94 (16%) in the *rad1* single mutant ($P=0.07$) and 44/95 (46%) in the *msh2* single mutant ($P<0.0001$) (Figure 4A). As discussed more fully below, these findings are best explained by a model in which Msh2:Msh3 detect the formation of a large unpaired

bubble during TGR and direct nicking of the targeting DNA by Rad1, leading to preferential repair of the bubble mismatch in favor of the chromosomal sequences (Figure 2B).

Patterns of flanking markers support the model of an alternate mechanism for TGR in the absence of RAD1

Previously, it was shown that TGR in wild-type yeast occurs by invasion of separate strands of targeting DNA at the two ends, followed by distinct, apparently uncoordinated processing events at the 5' and 3' ends of a given strand (Langston and Symington, 2004) (Figure 2A). A model to account for TGR in the absence of Rad1 is that the process frequently occurs by assimilation of a single strand of targeting DNA into a long, unrepaired hDNA tract encompassing portions of both flanking homology regions (Figure 2D). A detailed analysis of *MET17* transformants in *rad1* and *msh2 rad1* shows that the majority of transformants incorporated the flanking marker from one end of the targeting DNA but not the other (Figure 5). By contrast, at *TRP1*, more than two-thirds of transformants incorporated both flanking markers in the *rad1* mutant ($P<0.0001$). The flanking markers on the *MET17* targeting fragment are much farther apart (2.2 kbp; Figure 1B; depicted by circles in Figure 2) than those on the *TRP1* targeting fragment (0.9 kbp; Figure 1A; depicted by boxes in Figure 2) and are less likely to be found in a common hDNA tract. These results strongly support a model for TGR in the absence of *RAD1* in which strand invasion by one 3' end of the targeting DNA is accompanied by heteroduplex extension sufficient to incorporate some or all of the 5' flanking sequences from the same strand, as shown on the right side of Figure 2. Sequences more distant from the invading end are less likely to be incorporated into the single hDNA tract, especially if the opposite, 5' end of the invading strand has undergone nucleolytic processing analogous to that at a DSB (depicted by dotted lines in Figure 2).

RAD1 is not required for correction of a point mutation by gene targeting

In TGR, the two ends of the linear targeting fragment are normally identical in sequence to the chromosomal regions flanking the targeted gene, but the gene itself is replaced by a selectable marker. Thus, after strand invasion, base pairing between the targeting DNA and the targeted gene can extend only as far as the homology/heterology boundary represented by the selectable marker. To explain the requirement for *Ercc1* during TGR in mammalian cells, Niedernhofer *et al* (2001) suggested that the *Ercc1:Xpf* endonuclease cleaves at the junction of homologous and heterologous sequences formed

Figure 5 Evidence for single-strand assimilation in the *rad1* strain. For the indicated strains, the data from the bars marked 'vector sites, no hDNA' in Figure 3 are analyzed further to show the percentage of colonies that contained the vector restriction site at only one flanking marker (left) or both flanking markers (right).

by strand invasion and branch migration (Niedernhofer *et al*, 2001). If this model is correct, deletion of *ERCC1* or *XPF* (*RAD10* or *RAD1*) should have no effect when the targeting DNA and the targeted gene are nearly identical.

To test this hypothesis, we examined the role of *RAD1* during gene targeting of a chromosomal frameshift mutation, *met17-sna*, using the *MET17* targeting fragment (Figure 1C). Like TGR, this type of gene targeting is initiated by separate strand invasions at the two ends in wild-type cells (Langston and Symington, 2004). As shown in Figure 6, deletion of *RAD1* had no significant effect on hDNA formation at *met17-sna* ($P=0.37$), and all eight transformants recovered from the *rad1* strain with hDNA in both flanking homology regions were in the *trans* configuration. Furthermore, deletion of *RAD1* had no effect on targeting frequency at *met17-sna* (data not shown), all of which suggest that correction of small point mutations by gene targeting occurs in a *RAD1*-independent manner. These results differ greatly from those during TGR at *met17::ADE2* and *trp1::URA3*, where deletion of *RAD1* had a significant effect on both hDNA formation and targeting frequency (Figures 3 and 4), suggesting that *RAD1* is only required during TGR when replacing a segment of chromosomal DNA with a large heterologous sequence.

The frequency of TGR increases significantly in the absence of the SGS1 gene with no apparent change in the mechanism of TGR

The RecQ family helicase Sgs1 was recently shown to suppress crossover recombination during DSB repair in yeast, possibly through a Holliday junction dissolution mechanism (Ira *et al*, 2003). With separate strand invasion at each end as the initiating event of TGR, integration of recombinant DNA into the genome requires covalent joining of both the invading and noninvading strands of targeting DNA to the

Figure 6 hDNA formation during TGR at *met17-sna* locus is unaffected by deletion of *RAD1*. hDNA formation and subclone analysis, as in Figure 4, for targeting of a chromosomal frameshift mutation, *met17-sna* (Figure 1C), are presented.

chromosomal DNA. In principle, one strand at each end could be connected through a concerted breakage and rejoining reaction like that postulated to occur during resolution of a Holliday junction. To determine whether Sgs1 plays a role in reversing branched intermediates formed during TGR, we determined the frequency of TGR at the *MET17* locus in the presence and absence of the *SGS1* gene. In the *sgs1* strain, TGR was almost six-fold higher than in the *SGS1* control ($P=0.0005$), showing that the suppression of mitotic crossover recombination by Sgs1 extends to TGR (Figure 3A).

To determine if this increase was attributable to an altered mechanism of TGR, we examined hDNA formation at the *MET17* locus in the *sgs1* strain. There was a modest although statistically significant reduction in hDNA formation, with 47 of 94 transformants (50%) showing hDNA at either end in the *sgs1* strain compared to 65/94 (69%) in *SGS1* ($P=0.01$; Figure 4A). However, among 14 transformants in the *sgs1* strain that showed hDNA in both flanking homology regions, subclone analysis showed that all 14 were in the *trans* configuration, as in the *SGS1* control (Figure 4A, bottom), suggesting that TGR is still initiated by separate strand invasions at the two ends of the targeting DNA in the absence of Sgs1. Thus, the increase in TGR frequency seen in the strain deleted of *sgs1* does not appear to be attributable to use of an alternate TGR pathway.

Discussion

This study was designed to investigate the mechanism of gene targeting and how it differs from other recombination processes. Although TGR efficiency varies widely in different organisms, it is extremely productive and efficient in *S. cerevisiae*; however, the basis for this proficiency, like the mechanism of TGR itself, is poorly understood. It was previously established that TGR in wild-type yeast and mammalian cells is initiated by separate strand invasions at the two ends of the targeting DNA (Figure 2A) (Li *et al*, 2001; Langston and Symington, 2004) and not by single-strand assimilation as suggested earlier (Leung *et al*, 1997). However, even though the early steps in TGR are the same, yeast and mammals differ greatly in the apparent requirement for their respective homologous Rad1:Rad10 and Ercc1:Xpf complexes, the latter of which is essential for TGR in mammals (Niedernhofer *et al*, 2001). As discussed below, we find that Rad1 is also important for two-end invasion in yeast but, paradoxically, deletion of *RAD1* potentiates TGR by the single-strand assimilation pathway, so the effect of *RAD1* deletion appears less severe in yeast than in mammals.

Msh2:Msh3 and Rad1 form the boundary between two distinct mechanisms of TGR

If single-strand assimilation occurs during TGR in wild-type cells, it is extremely unproductive because, among 44 transformants with hDNA in both flanking homology regions, only one was in the *cis* configuration (Langston and Symington, 2004). The best evidence that this mechanism is suppressed during TGR in wild-type cells comes from *msh2* and *msh3* mismatch repair mutants, where we find a significant shift to the *cis* configuration of flanking markers (Figure 4, bottom).

Assimilation of both flanking homology regions from a single strand of targeting DNA into a long hDNA tract with the homologous chromosomal locus results in a large bubble mismatch between the selectable marker on the targeting DNA and the targeted gene on the chromosome, as shown on the right-hand side of Figure 2. In wild-type cells, we suggest that Msh2 and Msh3 direct repair of the bubble mismatch in favor of the chromosomal sequences, so this pathway does not lead to productive TGR (Figure 2B). In *msh2* and *msh3* mutants, by contrast, the bubble mismatch is still repaired, but the bias in favor of chromosomal sequences is relaxed (Figure 2C), resulting in more transformants with the *cis* configuration of flanking markers (Figure 4, bottom). The fact that TGR frequency declines in *msh2* and *msh3* strains even though these mutations open up a secondary pathway for TGR strongly suggests that Msh2 and Msh3 facilitate targeted integration by the two-end invasion pathway in wild-type yeast (Figure 2, left) while simultaneously inhibiting the single-strand assimilation pathway.

Our data suggest a similar dual role for Rad1, but the effect of *rad1* mutation is a highly significant reduction in the proportion of flanking marker hDNA rather than a shift to the *cis* configuration (Figure 4). Combined with a two- to three-fold reduction in TGR frequency in *rad1* mutants (Figure 3), the four- to 10-fold reduction in hDNA proportion is consistent with a significantly reduced ability to complete targeted integration by the two-end invasion pathway in the absence of Rad1. As with *msh2* and *msh3*, we suggest that the bulk of TGR in *rad1* mutants occurs by single-strand assimilation; however, unlike *msh2* and *msh3*, *rad1* mutants are unable to repair large bubble mismatches formed by the single-strand assimilation pathway (Figure 2D). Thus, after the next round of DNA replication and cell division, one daughter cell fails to grow because it lacks the selectable gene on the targeting DNA leading to the extremely low levels of hDNA (and the lack of *cis* transformants) observed in *rad1* (Figure 4). The failure of *rad1* mutants to repair large bubble mismatches has previously been shown during meiotic recombination (Kirkpatrick and Petes, 1997; Kearney *et al*, 2001), but this is the first evidence for a similar role in large loop repair during mitotic recombination.

Although other explanations are possible for the observed reduction in hDNA, several results support the hypothesis that deletion of *RAD1* shifts the balance of TGR from the two-end invasion pathway to the single-strand assimilation pathway. First, as shown in Figure 6, the defect in hDNA formation in *rad1* mutants is specific to TGR with a heterologous selectable marker and not a general defect in heteroduplex formation. Second, as indicated by the boxes and circles in Figure 2, the single-strand assimilation model for TGR predicts that markers near the homology/heterology boundary are more likely to be found in a common hDNA tract, and the data presented for *rad1* mutants in Figure 5 support this expectation. Finally, we previously examined repair of a single base-pair insertion by gene targeting and found that *PMS1* prevents single-strand assimilation in this context. As with the *rad1* mutants presented here, deletion of *PMS1* was accompanied by a reduction in flanking marker hDNA, consistent with the model presented in Figure 2D (Langston and Symington, 2004).

How do Msh2:Msh3 and Rad1 facilitate TGR in wild-type cells?

The dual activities of Msh2:Msh3 and Rad1 in TGR are strongly reminiscent of the role these same factors play in other types of recombination involving interrupted homology sequences. For example, recombination between directly repeated sequences by single-strand annealing (SSA) leaves unpaired 3'-ended sequences that are stabilized by Msh2:Msh3 and cleaved by Rad1:Rad10 to permit completion of recombination (Schiestl and Prakash, 1988, 1990; Saparbaev *et al.*, 1996). Rad1 and Msh2:Msh3 are also required for the removal of terminal heterologies during Rad51-dependent strand invasion (Fishman-Lobell and Haber, 1992; Sugawara *et al.*, 1997). Similarly, in TGR, annealing between the targeting DNA and the targeted chromosomal locus is limited to the flanking homology sequences and pairing does not occur between the selectable marker and the targeted gene. Rad1 is not required when correcting a single base-pair insertion mutation by gene targeting (Figure 6), so clearly the requirement for Rad1 in TGR is related to the interrupted homology, but its exact role is unclear.

Based on the known substrate specificities of the Rad1:Rad10 and Ercc1/Xpf endonucleases (Bardwell *et al.*, 1994; Davies *et al.*, 1995; de Laat *et al.*, 1998), there are various ways that Rad1 may be acting to facilitate TGR, as shown in Figure 7. Although data presented here support the hypothesis of Niedernhofer *et al.* (2001), that these enzymes act at the homology/heterology border, replacement of the targeted locus requires cleavage at all four positions indicated in Figure 7A or additional processing by unidentified factors to remove the intervening chromosomal DNA. An alternative hypothesis, presented in Figure 7B, involves pairing of the D-loop with the noninvading strand of targeting DNA, resolution of the Holliday junction, and cleavage of the D-loop by Rad1:Rad10, as shown. While an enzyme for processing Holliday junctions has yet to be identified in eukaryotes, a resolvase activity with specificity similar to that of *E. coli* RuvC has been demonstrated in fractionated extracts of mammalian cells (Constantinou *et al.*, 2001, 2002), and its ultimate identification should allow us to examine its role in TGR. An alternate pathway for gene targeting that is independent of the need for Holliday junction resolution has been suggested involving strand invasion and duplication of the entire donor chromosome onto the two ends of the targeting fragment (Morrow *et al.*, 1997). It is unclear how this pathway would contribute to TGR because the targeted locus is not altered by break-induced replication (BIR). Furthermore, RAD51 is required for BIR (Davis and Symington, 2004) but is less important for gene targeting (Schiestl *et al.*, 1994) and *rad1* mutants are defective for TGR but not for BIR (A Davis and LS Symington, unpublished data).

Finally, as shown in Figure 7C, homologous integration of one end of targeting DNA into the chromosome creates two chromosome fragments with directly repeated sequences at both free ends, as in SSA. Repair could proceed by SSA using the overlapping homologies present on the two chromosome fragments. Because the regions of homology are interrupted, a branched structure would be formed at the homology/heterology boundary requiring the Rad1:Rad10 nuclease for resolution. Thus, Rad1 might not be required for the initial integration but for later steps in repairing the resulting DSB.

Figure 7 Possible roles for the Rad1:Rad10 nuclease in TGR. (A) Two different positions at the homology/heterology boundary where Rad1:Rad10 could nick the chromosome to facilitate TGR are indicated by arrows labeled 1 and 2. (B) D-loop pairs with noninvading strand; Rad1:Rad10 (black arrows) works in combination with a Holliday junction resolvase (open arrows). (C) One end of targeting DNA integrates into the chromosome in the flanking homology region labeled D, creating two chromosome fragments; 5'-end resection of both fragments reveals homologous sequences at D and F, interrupted by heterologous sequences at E and G; processing by Rad1:Rad10 at the homology/heterology boundary (indicated by an arrow) leads to TGR. For clarity, centromeric and telomeric positions on the targeted chromosome arm are indicated by open circles and flanges, respectively.

Deletion of Sgs1 increases TGR frequency by the two-end invasion pathway

Sgs1 was recently shown to suppress crossovers during DSB repair, possibly by a Holliday junction dissolution mechanism (Ira *et al.*, 2003), and it also cooperates with Msh2 and other mismatch repair factors to inhibit recombination between diverged sequences (Spell and Jinks-Robertson, 2004; Sugawara *et al.*, 2004). In the present study, deletion of *SGS1* led to a significant increase in the frequency of gene replacement (Figure 3A) without a major alteration in the mechanism of TGR (Figure 4A), which is the opposite of *msh2* mutants. Thus, it is likely that Sgs1 does not inhibit TGR as part of a heteroduplex rejection complex but by unwinding recombination structures formed in the homologous flanking regions. Whether these structures are D-loops or Holliday junctions remains to be determined, as both intermediates are substrates for the mammalian RecQ homolog *BLM* (Karow *et al.*, 2000; van Brabant *et al.*, 2000). Because a similar increase in TGR was previously identified in human and chicken cells deficient in *BLM* (Wang *et al.*, 2000; Traverso *et al.*, 2003) and *WRN* (Imamura *et al.*, 2002), it appears that deletion or inhibition of RecQ helicases may be a

general technique for improving gene targeting efficiency that can be applied to a wide variety of cell types. These results also confirm strong similarities between yeast and mammals in the early steps of TGR.

We now have a developing picture of the means by which linear targeting DNA containing a selectable marker is precisely integrated into the yeast chromosome at a position determined by the flanking homology sequences on the targeting DNA. It is clear that TGR is initiated by strand invasion into homologous sequences by at least one end of the targeting fragment and that this strand invasion is actively opposed or unwound by Sgs1. In most cases, heteroduplex extension of the initial strand invasion intermediate is blocked by the heterology between the selectable marker and the targeted locus, creating a requirement for Msh2:Msh3 and Rad1:Rad10 for further processing, although the exact nature of this processing is unclear. The heterology block also necessitates a second strand invasion by the opposite end of the targeting fragment to complete TGR by this process, known as two-end invasion/resolution (Figure 2, left).

In some cases, the hDNA tract initiated by the first strand invasion extends far enough to include the opposite flanking homology sequence, incorporating the selectable marker and the targeted locus into a large bubble heteroduplex (Figure 2, right). This process, known as single-strand assimilation, is normally reversed by the concerted repair activity of Msh2:Msh3 and Rad1 (Figure 2B) and thus is rarely observed in wild-type cells. Formation and repair of large insertion/deletion mispairs have also been demonstrated in mammalian cells (Raynard and Baker, 2002), but repair of a preformed 216-nucleotide insertion mispair in mammalian cell extracts was independent of Ercc1/Xpf (Littman *et al.*, 1999), which might explain why this pathway is unavailable for TGR in *Ercc1*^{-/-} ES cells.

Materials and methods

Yeast strains and plasmids

The *S. cerevisiae* strains used for this study are derivatives of haploid strain W303-1A (Thomas and Rothstein, 1989) with the corrected *RAD5* allele (Fan *et al.*, 1996) and are listed in Table I. Parental strains used for gene targeting assays, LSY697, LSY698, and LSY1103, containing the *met17-sna*, *met17::ADE2*, and *trp1::URA3* alleles, respectively, were described previously (Bartsch *et al.*, 2000; Langston and Symington, 2004). Strains containing

msh2::HIS3, *msh3::HIS3*, *msh6::TRP1*, and *mus81::kanMX6* alleles were made by PCR-mediated gene disruption (Baudin *et al.*, 1993) of LSY1103 or LSY698, and correct integration was confirmed by PCR or Southern blot. Strains containing *msh2::URA3* were derived from crosses to HKY592-11A (a gift from Hannah Klein, New York University School of Medicine, New York). LSY1683 was derived from a cross between LSY698 and W3643-38A (a gift from Rodney Rothstein, Columbia University, New York).

Plasmids pLL113 (*ARS1*⁺) and pLL147 used in gene targeting assays were described previously (Langston and Symington, 2004). The *TRP1* linear targeting fragment containing diagnostic restriction site polymorphisms in the flanking homology regions was released from pLL113 by digestion with restriction endonucleases *SspI* and *BsaBI*. The *MET17* linear targeting fragment, also containing flanking restriction site polymorphisms, was released from pLL147 by *SpeI* digestion. Uncut transformation control plasmids pSB110 and pRS424, containing the *MET17* and *TRP1* genes, respectively, were described previously (Sikorski and Hieter, 1989; Bartsch *et al.*, 2000).

Determination of transformation frequency and analysis of hDNA

Transformation of competent yeast cells was carried out by the lithium acetate method using 100–300 ng of linear targeting fragment DNA or 50–100 ng of uncut plasmid control DNA along with 50 µg of salmon sperm carrier DNA. Transformed cells were plated onto synthetic complete (SC) medium lacking Trp (SC–Trp) or Met (SC–Met), as appropriate. To exclude transformants arising by circularization of the linear targeting fragment or random integration, Trp⁺ colonies were replicated to medium containing 5-fluoroorotic acid (5-FOA) to assure TGR of the chromosomal *trp1::URA3* marker. Similarly, Met⁺ colonies were replicated to medium lacking both Met and Ade (SC–Met–Ade) to assure TGR of the chromosomal *met17::ADE2* marker.

To determine the frequency of TGR for each strain, colony-forming units (CFU) per 100 ng of transformed linear targeting DNA were compared to CFU/1 ng for the corresponding uncut plasmid for each strain (Figure 3) to normalize for differing transformation competence among strains. Transformation efficiencies at *TRP1* were approximately 10-fold lower than those at *MET17*, reflecting normal variation in transformation efficiencies with different selectable markers and at different locations in the yeast genome. Except where noted, each transformation was performed at least three times and the means and standard deviations from all trials are shown in Figure 3. Statistical comparisons of mean TGR efficiencies between strains were performed using the unpaired *t*-test. *P*-values < 0.05 were considered statistically significant.

Analysis of hDNA was previously described in detail (Langston and Symington, 2004). Briefly, genomic DNA from ~96 whole colonies arising from TGR of each strain was amplified by PCR and digested separately with restriction enzymes corresponding to the restriction site on the targeting vector DNA or the site on the chromosomal DNA. PCR products that digested to completion with the enzyme specific to the restriction site on the targeting vector

Table I Yeast strains

Strain no.	Genotype ^a	Source or reference
LSY697	<i>met17-sna ADE2</i>	Bartsch <i>et al.</i> (2000)
LSY698	<i>met17::ADE2</i>	Bartsch <i>et al.</i> (2000)
LSY928-9C	<i>met17::ADE2 rad1::LEU2</i>	Symington <i>et al.</i> (2000)
LSY960-8B	<i>met17::ADE2 msh2::URA3</i>	This study
LSY1103	<i>trp1::URA3</i>	Langston and Symington (2004)
LSY1187	<i>trp1::URA3 msh2::HIS3</i>	This study
LSY1256	<i>trp1::URA3 rad1::LEU2</i>	This study
LSY1512	<i>met17::ADE2 msh3::HIS3</i>	This study
LSY1513	<i>met17::ADE2 rad1::LEU2 msh2::URA3</i>	This study
LSY1542	<i>met17::ADE2 msh6::TRP1</i>	This study
LSY1570	<i>met17-sna rad1::LEU2 ADE2</i>	This study
LSY1571	<i>met17::ADE2 mus81::kanMX6 rad1::LEU2</i>	This study
LSY1683	<i>met17::ADE2 sgs1::HIS3</i>	This study

^aAll strains are derived from LSY678, a *RAD5* derivative of haploid strain W303-1A (*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 rad5-535*) (Thomas and Rothstein, 1989); only differences from this genotype are given.

DNA or the site on the chromosomal DNA were scored as 'vector site only' or 'chromosomal site only', respectively. PCR products that showed partial digestion with both enzymes were scored as hDNA. For Figure 4 (top), if either flanking homology region showed hDNA, the colony is shown as 'hDNA either end'; if neither flanking region showed hDNA but at least one region contained vector sequences, the colony is represented as 'vector site(s), no hDNA'; if both flanking regions showed only chromosomal sequences, the colony is represented as 'chromosomal sites only'. To compare hDNA formation, differences between strains in the number of hDNA and non-hDNA transformants were treated as categorical variables and analyzed by Fisher's two-tailed exact test. *P*-values < 0.05 were considered statistically significant. Colonies that showed hDNA in both flanking homology regions were

subcloned and analyzed by PCR and restriction digestion to determine if hDNA formed between the same strand (*cis*) or different strands (*trans*) of the targeting DNA at the two ends, and the results are shown at the bottom of Figure 4.

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