Gene targeting in yeast is initiated by two independent strand invasions

Lance D. Langston* and Lorraine S. Symington†‡

*Integrated Program in Cellular, Molecular, and Biophysical Studies and †Department of Microbiology, Columbia University Medical Center, 701 West 168th Street, New York, NY 10032

To study the mechanism of gene targeting, we examined heteroduplex DNA (hDNA) formation during targeting of two separate chromosomal locations in Saccharomyces cerevisiae. We examined both replacement of the entire gene with a heterologous selectable marker and correction of a single base pair insertion mutation by gene targeting, and in all cases our results were consistent with separate strand invasion/resolution at the two ends of the targeting fragment as the dominant mechanism in wild-type cells. A small subset of transformants was consistent with assimilation of a single strand of targeting DNA encompassing both flanking homology regions and the marker into hDNA. hDNA formation during correction of a point mutation by targeted integration was conspicuously altered in a mismatch repair-deficient background and was consistent with single-strand invasion/assimilation without mismatch correction, confirming that gene targeting by this pathway is actively impeded in wild-type yeast. Finally, inversion of one targeted locus and mutation of an active origin of DNA replication at the other locus affected hDNA formation significantly, suggesting that formation of productive interactions between the targeting DNA and the targeted site in the chromosome is sensitive to local DNA dynamics.

Targeted gene disruption was introduced in the late 1970s and since has become one of the most useful methods available for investigating gene function. In early studies in yeast, genes were disrupted by inserting a piece of the targeted gene into a plasmid to facilitate homologous recombination of the foreign plasmid DNA into the yeast chromosome (1, 2). Subsequently, it was found that cutting the plasmid in the region of homology to the chromosome greatly increased the frequency of recombination in both yeast (3) and mammalian (4, 5) cells. This finding led to the proposal that this type of gene disruption, known as “ends-in” gene targeting, occurs by a process analogous to double-strand break repair (DSBR), whereby integration of the broken plasmid into the chromosome occurs during repair of the break by gene conversion associated with crossing over (3).

The ends-in method disrupts gene function by inserting foreign DNA at the target site without deleting the targeted gene.

To address the limitations of insertional mutagenesis, gene replacement strategies were developed in yeast (6) and later implemented in mammalian cells (7) by using recombinant linear DNA in which the two ends of the fragment are homologous to the regions flanking the targeted gene but the gene itself is replaced by a selectable marker. The ends of the linear targeting fragment are recombinogenic, facilitating replacement of the targeted gene with the selectable marker. This type of gene targeting is called “ends-out” because the edges of the targeting DNA correspond to two divergent, discontinuous stretches of chromosomal DNA. In effect, the two ends of the targeting fragment are separate, unrelated broken ends, so the mechanism of integration probably involves initially uncoordinated interactions between each end of the targeting fragment and its corresponding region of homology in the chromosome. Thus, integration of ends-out targeting DNA must occur by some mechanism other than simple gene conversion accompanied by crossing over.

This notion is reinforced by the observation that, whereas mitotic gene conversion is reduced 50- to 100-fold in mutants in which the Rad51 recombibnase is absent (8, 9), targeted integration frequency is reduced only ∼8-fold (10). Despite limited dependence on RAD51, the mechanism of ends-out gene targeting in yeast clearly requires the broader recombination machinery because targeted integration is extremely deficient in rad52 mutants (10). This observation at first glance seems paradoxical, because the primary role of Rad52 in recombination is generally believed to be accessory to Rad51, but Rad52 also participates in a variety of RAD51-independent recombination events (reviewed in ref. 11). In vitro, Rad52 anneals complementary oligonucleotides (12), suggesting a possible role for this protein in base-pairing one strand of the targeting DNA with the targeted chromosomal locus in the absence of Rad51.

Despite these obvious differences from DSBR, it has been postulated that ends-out gene targeting occurs by separate crossovers at the two ends of the targeting fragment (13), but supporting data are limited and important mechanistic questions remain unanswered. Li et al. (14) examined heteroduplex DNA (hDNA) formation during targeted gene replacement in a mouse hybridoma cell line and found evidence of hDNA in both flanking homology regions in 9 of 26 transformants, eight of which were consistent with the model featuring invasion of the chromosome by different strands of targeting DNA at the two ends, as shown in Fig. 1a. Leung et al. (15) raised the idea that gene targeting can occur by assimilation of a single strand of the targeting fragment into heteroduplex with the corresponding chromosomal locus followed by mismatch correction (Fig. 1b).

However, clear evidence for this mechanism was found only in mismatch repair-defective pms1 mutants, not in wild-type cells. Furthermore, targeting fragments used in that study (15) differed from the targeted chromosomal locus by only a few base pairs, not the large central heterologies typical of targeted gene replacement. It appears that even when single-strand assimilation intermediates were able to form, subsequent mismatch repair was overwhelmingly in favor of the chromosomal allele. Thus, gene targeting by this mechanism would be unlikely in wild-type cells. A separate study in yeast showed large increases in targeted gene replacement in mutants from which a different mismatch repair gene, MSH2, was deleted (16), suggesting that the mismatch repair machinery in wild-type cells acts specifically to prevent integration of a selectable marker, even when flanked by large regions of homology.

To investigate the mechanism of gene targeting in wild-type yeast, we developed targeting fragments that allow us to physically examine transformants for evidence of hDNA in the flanking homology regions during targeted integration. Our results suggest that gene targeting is typically initiated by separate strand invasions at the two ends of the targeting molecule.

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2Abbreviations: hDNA, heteroduplex DNA; DSBR, double-strand break repair.
3To whom correspondence should be addressed. E-mail: lss5@columbia.edu.
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Materials and Methods

Yeast Strains. All strains are derived from LSY678, a RAD5 derivative of W303-1A (17). Strains LSY697 and LSY698, containing the met17-sna and met17::ADE2 alleles, respectively, have been described previously (8). To create a trp1::URA3 derivative, LSY678 was transformed with a SnaBI/ApaII fragment from plasmid pRS414 (18), creating strain LSY1099. The TRP1 ORF of LSY1099 was replaced with the URA3 marker by PCR-mediated gene disruption (19) to create strain LSY1103. TRP1-ar51 - strain LSY1508 was made by transforming LSY1103 with an SpeI/BsaBI fragment from pLL111-ar51 - . Trp+ Ura- clones were checked by PCR for the presence of a Xhol mutation at ar51. LSY1509, a trp1::URA3-ar51 - derivative of LSY1508, was made by PCR-mediated gene disruption of LSY1508 using the same primers used to make LSY1103. Inverted met17::ADE2 strain LSY1539 was created by transforming LSY678 with BstEII-digested pLL152, and the inversion was confirmed by PCR. LSY946-1C was made by crossing LSY697 to pms1 :: trp1 strain HKY594-1B (a gift from Hannah Klein, New York University School of Medicine, New York).

Plasmids. Plasmid pLL140 contains a 3.3-kb MET17 fragment cloned into plasmid pGEM-T (Promega). Plasmid pLL147 is a derivative of pLL140 with the following interrupted palindromic inserts cloned into unique restriction sites in the MET17 flanking regions: upstream insert at SwaI, 5'-ATTTTctagctagcttttaggtcagctgTA3'; downstream insert at BglII, 5'-Acgtcgatccggattttttccggatc-ggATCT-3'.

Plasmid pLL111 contains a 2.2-kb TRP1 fragment cloned into plasmid pGEM-T. Plasmid pLL113 is a derivative of pLL111 with the following interrupted palindromic inserts cloned into unique restriction sites in the TRP1 flanking regions: upstream insert at SnaBI, 5'-TACgctagctatcctttaggtcagctgGT3'; downstream insert at BglII, 5'-AcgtcgatccgatcggatcttttaggtcagctgGTA-3'.

An ars1 mutant derivative of pLL111, pLL111-ars1 -, was made by replacing the A site with a XhoI linker by site-directed mutagenesis using the Gene Editor kit (Promega) (20). An ars1 derivative of pLL113, pLL113-ars1 -, was made by subcloning sequences from pLL113 into pLL111-ars1 -. Plasmids pLL153 and pLL154 were created by using the same primers and technique used to create pLL111, except that the template was genomic DNA from strains LSY1103 (trp1 :: URA3 and ARS1) and LSY1509 (trp1 :: URA3 and ars1 -), respectively. All plasmids containing ARS1 were able to confer high-frequency transformation of yeast whereas both ars1 - derivative plasmids were not, indicating a disruption of origin activity.

To invert the met17::ADE2 disruption, a 7.1-kb fragment containing met17::ADE2 from LSY698 was PCR-amplified and cloned into the NotI and BamHI sites of pRS423 (18), creating pLL151. To flip met17::ADE2, plasmid pLL151 was digested with SpeI and religated. Clones with the 2.9-kb SpeI fragment in inverted orientation with respect to the surrounding sequences were identified by restriction analysis and designated pLL152.

Analysis of hDNA. For gene targeting, plasmids pLL113 and pLL113-ars1 - were digested with SspI and BsaBI to release the TRP1 fragment, and plasmid pLL147 was digested with SpeI to release the MET17 fragment. Transformation of yeast cells was done by the lithium acetate method using ~100–300 ng of linear targeting DNA 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 end-joining of the fragment or random integration, Trp+ colonies were replica-plated to medium containing 5-fluoroorotic acid (5-FOA) to identify clones that had lost the trp1 :: URA3 marker. Similarly, Met+ transformants that had replaced the met17::ADE2 marker were identified by colony color or by replica plating to SC–Met–Ade. The Met+ Ade- transforms accounted for ~10% of total events. To exclude random integration events in the met17-sna background, genomic DNA from Met+ clones was amplified by PCR with primers flanking the met17-sna mutation. PCR products were digested with SnaBI, which cuts MET17 sequences but not met17-sna. PCR products that failed to digest to completion were not analyzed further. Transformation efficiencies of the different target strains were similar for a given
marker (data not shown) except for the pms1 strain, as described in Results. The transformation efficiency at TRP1 was ~10-fold lower than at MET17 (data not shown).

After identifying correct clones by phenotype, whole individual colonies were suspended in 5 ml of synthetic complete (SC) medium and grown overnight. Ten microliters of each culture was spotted onto selective media to preserve the strains for further analysis, and ~96 individual colonies were analyzed for each transformed strain. Genomic DNA from each strain was amplified by PCR with primers specific to the upstream or downstream flanking region and then digested with restriction enzymes diagnostic of the chromosomal allele or the targeting ORF that contains the promoter of the relevant gene; “downstream” refers to the region 5’ to the ORF that contains the promoter of the relevant gene; “downstream” refers to the region 5’ to the ORF that contains the transcriptional terminator of that gene. Cleavage of the PCR product with one enzyme but not the other identifies that locus as containing exclusively chromosomal or exclusively targeting vector DNA. Partial cleavage with both enzymes indicates that the particular transformant gave rise to a colony with a mixed population of cells resulting from failure to repair hDNA formed during the targeting process (referred to as “sectoring”).

Clones that were scored as hDNA in both flanking regions were subcultured to segregate the two cell types and isolate pure clonal populations. Six to eight individual subclones were characterized by PCR and restriction digestion. Two patterns of subclones were expected. In one, both the upstream and downstream PCR products from all subclones were cleaved by enzymes diagnostic of the chromosomal allele or the targeting ORF that contains the promoter of the relevant gene; “downstream” refers to the region 5’ to the ORF that contains the transcriptional terminator of that gene. Cleavage of the PCR product with one enzyme but not the other identifies that locus as containing exclusively chromosomal or exclusively targeting vector DNA. Partial cleavage with both enzymes indicates that the particular transformant gave rise to a colony with a mixed population of cells resulting from failure to repair hDNA formed during the targeting process (referred to as “sectoring”).

Targeted Gene Replacement Occurs by Invasion of Separate Strands at Each End. We first examined hDNA formation during targeted replacement of the chromosomal trp1::URA3 allele by TRP1 (Fig. 2a). Of 103 Trp+ Ura+ transformants examined, 58 were sectored for the upstream hDNA marker (56%), and 22 were sectored downstream (21%) (Fig. 3a). Of 82 transformants scored for the downstream flanking marker (26%) (Fig. 3b). Sixty-five transformants exhibited hDNA in one or both flanking regions (69%), and eight transformants (9%) showed evidence of hDNA in both flanking regions (Table 1). In this case, all eight double-sectoring transformants were in the trans configuration (Table 1) indicating that, at least during gene replacement in wild-type yeast, gene targeting is normally initiated by different strands of DNA at the two ends of the molecule and not by assimilation of a single strand into hDNA.

Results

Experimental System. We designed an experimental system that relies on restriction site polymorphisms to detect formation of heteroduplex between homologous regions of targeting DNA and the chromosome during gene targeting. To avoid mismatch repair of the resulting hDNA, the altered restriction sites were incorporated into 26-bp palindromic inserts that are poor substrates for mismatch repair (21).

After identifying transformants as isolated single colonies arising from targeted integration, we examined the regions of the flanking sequences containing the restriction site polymorphisms by PCR, digesting the PCR products with restriction endonucleases to distinguish chromosomal DNA from targeting DNA in the upstream and downstream flanking regions. Throughout this paper, “upstream” refers to the region 5’ to the ORF that contains the promoter of the relevant gene; “downstream” refers to the region 3’ to the ORF that contains the transcriptional terminator of that gene. Cleavage of the PCR product with one enzyme but not the other identifies that locus as containing exclusively chromosomal or exclusively targeting vector DNA. Partial cleavage with both enzymes indicates that the particular transformant gave rise to a colony with a mixed population of cells resulting from failure to repair hDNA formed during the targeting process (referred to as “sectoring”).

Clones showing hDNA formation in both the upstream and downstream flanking homology regions were further characterized by subclone analysis to determine whether the same or opposite strands of targeting DNA invaded the chromosome at the two ends, as shown in Fig. 1 (see Materials and Methods for details).

Statistical Analysis. For different strains targeted with the same selectable marker, differences between strains in the number of hDNA and non-hDNA transformants at either the upstream or downstream locus were treated as categorical variables and analyzed by Fisher’s two-tailed exact test. P values < 0.05 were considered statistically significant.

Fig. 2. Schematics of targeting DNA and targeted yeast strains. Open arrows show the selectable markers, TRP1 or MET17, black boxes show the corresponding disrupted allele in the chromosome, and open circles indicate the position of the centromere with respect to the targeted locus (not to scale). (a) Targeting at the TRP1 locus. (b) Targeting at the MET17 locus of a complete disruption (Upper) or a point mutation (Lower). Restriction site polymorphisms in the flanking homology regions are indicated.
selectable marker poses a barrier to gene targeting by single-strand invasion, we examined mutation correction by gene targeting using the met17-sna strain shown in Fig. 2b. In this case, the only difference between the MET17 sequence on the targeting DNA and the met17-sna allele on the chromosome is a single base pair insertion in an SnaBI site on the chromosomal allele, in addition to the palindromic insertions (8). Of 93 Met⁺ transformants shown to have replaced the met17-sna allele, 33 were sectored for the upstream flanking marker (35%), and 15 were sectored for the downstream flanking marker (16%) (Fig. 3c). Formation of hDNA was detected in at least one of the flanking regions in 39 transformants (42%), whereas nine transformants (10%) showed hDNA formation in both flanking regions (Table 1). Subclone analysis of these transformants showed seven and two in the trans and cis configurations, respectively (Table 1), suggesting that the mechanism of successful targeted integration does not differ significantly between gene replacement and mutation correction in wild-type cells (P = 0.47). These results do not exclude the possibility that single-strand invasion intermediates form in wild-type cells, but, if so, they are probably corrected to the chromosomal allele in most cases. In this way, our results are similar to those of Leung et al. (15), who found evidence for single-strand assimilation in mismatch repair-deficient pms1 mutants but not in wild-type cells.

**PMS1 Prevents Gene Targeting by Assimilation of a Single Strand.** To investigate the effects of mismatch repair, the gene targeting experiments at met17-sna were repeated in a strain deleted of PMS1, which encodes one of the four MutL homologs in Saccharomyces cerevisiae. We found a dramatic reduction in the number of transformants with hDNA in the pms1 strain, consistent with the formation, but not repair, of hDNA between a single strand of the MET17 targeting DNA and one strand of the chromosomal met17-sna locus (Fig. 1c). Replication of the unrepaired intermediate would yield only one daughter cell with the MET17 allele, resulting in an unsectored colony. Of 96 Met⁺ transformants shown to have replaced the met17-sna allele, only three were sectored for the upstream flanking marker (3%), three were sectored for the downstream flanking marker (3%) (Fig. 3c), and none of the transformants analyzed showed sectoring for both flanking markers. Along with the changes in hDNA formation, we also observed a >3-fold increase in the number of Met⁺ Ade⁻ transformants in the pms1 strain (data not shown).

To create a MET17 strain by single-strand assimilation, the hDNA tract initiated by 3′ end invasion of the targeting DNA has to extend at least as far as the met17-sna mutation. The mutation in met17-sna is much closer to the downstream flanking marker than to the upstream flanking marker (Fig. 2b). Thus, if targeting occurs by single-strand assimilation without mismatch correction in the pms1 strain, we would expect the downstream flanking marker to be in hDNA with met17-sna more often than the upstream marker. Vector sequences were found at the downstream locus in 49 of 96 transformants (51%) but in only 23 of

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**Table 1. Summary of hDNA and subclone analysis**

<table>
<thead>
<tr>
<th>Targeting DNA/targeted strain</th>
<th>hDNA, %</th>
<th>Either end</th>
<th>Both ends</th>
<th>No. in trans</th>
<th>No. in cis</th>
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<td>TRP1/trp1::URA3 (ARS1⁺)</td>
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<td>10</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>TRP1-ars1⁺/trp1::URA3 (ars1⁻)</td>
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<td>7</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>MET17/met17-::ADE2</td>
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<td>69</td>
<td>9</td>
<td>8</td>
<td>0</td>
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<tr>
<td>MET17/inverted met17-::ADE2</td>
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<td>20</td>
<td>19</td>
<td>0</td>
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<tr>
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<td></td>
<td>42</td>
<td>10</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>MET17/met17-sna pms1</td>
<td></td>
<td>6</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

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**Fig. 3.** hDNA formation during gene targeting. hDNA was analyzed by restriction analysis after gene targeting of each indicated strain. The number of transformants analyzed in each strain is indicated. Each stacked bar indicates the percentage of transformant colonies containing only the chromosome-specific (Bottom, vector site only), only the targeting vectorspecific (Middle, vector site only), or a mixture of both (Top, hDNA) restriction sites in the upstream or downstream flanking regions. (a) Gene replacement at the TRP1 locus. (b) Gene replacement at the MET17 locus. (c) Mutation correction by gene targeting at the MET17 locus.

**Targeting of a Single Base Pair Mutation also Occurs by Separate Strand Invasions.** To determine whether gene replacement with a selectable marker poses a barrier to gene targeting by single-strand invasion, we examined mutation correction by gene targeting using the met17-sna strain shown in Fig. 2b. In this case, the only difference between the MET17 sequence on the targeting DNA and the met17-sna allele on the chromosome is a single base pair insertion in an SnaBI site on the chromosomal allele, in addition to the palindromic insertions (8). Of 93 Met⁺ transformants shown to have replaced the met17-sna allele, 33 were sectored for the upstream flanking marker (35%), and 15 were sectored for the downstream flanking marker (16%) (Fig. 3c). Formation of hDNA was detected in at least one of the flanking regions in 39 transformants (42%), whereas nine transformants (10%) showed hDNA formation in both flanking regions (Table 1). Subclone analysis of these transformants showed seven and two in the trans and cis configurations, respectively (Table 1), suggesting that the mechanism of successful targeted integration does not differ significantly between gene replacement and mutation correction in wild-type cells (P = 0.47). These results do not exclude the possibility that single-strand invasion intermediates form in wild-type cells, but, if so, they are probably corrected to the chromosomal allele in most cases. In this way, our results are similar to those of Leung et al. (15), who found evidence for single-strand assimilation in mismatch repair-deficient pms1 mutants but not in wild-type cells.

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96 (24%) at the upstream locus ($P = 0.0002$) (Fig. 3c). Trans- 
formants incorporating both flanking markers were rare (8 of 96, 
or 8%; data not shown), because this requires both that the 
DNA tract be long (at least 2.2 kb) and that nucleosome 
processing of the 5′ end of the assimilated strand be limited to <100 or 165 
bp, depending on which 3′ end invaded (Fig. 2b). Altogether, the 
data from the pms1 mutant show that the mechanism of gene 
targeting of a point mutation is significantly altered in the 
absence of at least one component of the mismatch repair 
machinery in a way that is consistent with single-strand invasion/ 
assimilation without mismatch correction (Fig. 1c).

Local DNA Dynamics Affect Interactions Between Targeting DNA and 
Targeted Locus. As shown in Fig. 3, hDNA formation at both 
TRP1 and MET17 was much greater in the upstream, promoter- 
proximal flanking region than in the downstream flanking 
region in wild-type cells. Both genes are transcribed in the same 
direction with respect to the centromere, so there were no clues 
as to whether the difference in hDNA formation up- 
and downstream at both loci was due to transcription or to some 
other factor, possibly the direction of replication fork movement 
through the locus or gross chromosome architecture. 
To examine this question, we inverted the chromosomal 
met17::ADE2 allele on a 2.9-kb SpeI fragment containing the 
ADE2 ORF under control of the MET17 promoter and flanked 
by ~600 bp of MET17 sequences on both sides (see Materials and 
Methods). Transformation was repeated by using the same 
MET17 targeting DNA as in the earlier experiments to replace 
the inverted met17::ADE2 allele. As shown in Fig. 3b, hDNA at 
the upstream flanking marker was essentially unchanged, with 42 
transformants showing hDNA of 95 total Met⁺ Ade⁻ trans- 
formants (44%), compared with 49 of 94 (52%) at the original 
met17::ADE2 locus ($P = 0.31$). In contrast, a small but signif- 
icant increase in hDNA was observed at the downstream flan- 
kling region, with 38 sectors of 95 transformants (40%) compared 
with 24 of 94 (26%) in the original orientation ($P = 0.044$) (Fig. 
3b). As a result, the difference in hDNA formation between the 
upstream and downstream flanking regions disappeared com- 
pletely. Inversion of the met17::ADE2 locus did not greatly 
affect its transcription, because ADE2 mRNA levels in the 
inverted strain were 60% of those in the noninverted strain as 
determined by Northern blot analysis (data not shown). This 
outcome suggests that the difference between the two flanking 
regions in hDNA formation observed with the original 
met17::ADE2 allele was not caused by transcription. Finally, 
because of the higher rate of hDNA formation downstream with 
the inverted met17::ADE2 allele, the proportion of transfor- 
mants showing hDNA in both flanking regions increased to 20% 
(Table 1). Subclone analysis of these transformants showed that 
all 19 were in the trans configuration (Table 1). 
To determine whether the origin of DNA replication (ARS1) 
at the TRP1 locus had any effect on hDNA formation or the 
mechanism of targeted integration, we mutated the origin in a 
way that was previously shown to eliminate its ability to support 
plasmid replication (20). The same ars1 mutation was made in 
both the targeting fragment and the chromosome to avoid 
introducing additional heterologies between the two DNAs and 
to assure that neither the targeting DNA nor the targeted region 
was the site of replication initiation. ARS1 has previously been 
shown to be an active origin in the W303 yeast strain background 
used in our experiments (22), and its ability to support plasmid 
replication is not affected by the adjacent trpl::URA3 disruption 
(data not shown).

As shown in Fig. 3a, hDNA formation at the downstream 
flanking region was unaffected by the ars1 mutation. This is 
particularly noteworthy because the downstream hDNA marker 
at TRP1 is directly adjacent to the site of replication initiation at 
ARS1 (23). Thus, it was surprising to find a reduction in hDNA 
at the upstream flanking marker in the ars1 mutant, with 37 
sectors of 96 Trp⁺ Ura⁻ transformants (39%) compared with 58 
of 103 (56%) with the wild-type ARS1 ($P = 0.016$). This result 
suggests that the presence of an active origin of DNA replication 
affects hDNA formation during gene targeting but, in this case, 
only at the upstream flanking marker distant from the origin and 
not at the downstream flanking marker located near the site of 
gene replication initiation. Furthermore, the ars1 mutation did not 
affect the mechanism of targeted integration: all seven trans- 
formants that showed hDNA formation in both the upstream 
and downstream flanking regions were in the trans configuration 
(Table 1).

Discussion

Several important differences between DSBR and gene target- 
ing led us to investigate the mechanism of targeted gene 
replacement in yeast. Furthermore, conflicting data from yeast 
and mouse cells raised the possibility of a fundamental differ- 
ence between these organisms that might explain the higher 
efficiency of homology-directed gene targeting in yeast. In 
particular, it was suggested that gene targeting in yeast might 
frequently occur by assimilation of a single strand of the target- 
ing DNA into heteroduplex with the homologous chromosomal 
locus, followed by mismatch correction in favor of the targeting 
DNA, as shown in Fig. 1b (15). However, evidence for such a 
mechanism was found only in pms1 mutants, and the targeting 
constructs in that study differed from the targeted locus by just 
a few base pairs. Contrasting studies (e.g., 14) of gene replace- 
ment at a single locus in a mouse hybridoma cell line found little 
evidence for single-strand assimilation in mammalian cells.

Our studies focused on hDNA formation during gene target- 
ing in wild-type yeast. To maximize the generality of our 
conclusions, hDNA formation was assayed at two different 
chromosomal loci, and both gene replacement and mutation 
correction by gene targeting were examined. Of 44 transfor- 
mants where hDNA was detectable in both flanking regions in 
the gene replacement studies, only one of these was in the cis 
configuration (Table 1). Similarly, examination of mutation 
correction by gene targeting produced nine transformants 
wherein hDNA was detectable in both flanking regions, and only 
one of these were in the cis configuration (Table 1). No such 
transformants were identified in the corresponding pms1 mu- 
tant. Thus, gene targeting by single-strand assimilation appears 
not to be an important pathway in wild-type yeast, even in the 
absence of large heterologies between targeting and chromo- 
somal DNA. Furthermore, it appears that gene replacement 
impaired repair-proficient mammals and yeast is initiated by 
invasion of the homologous chromosomal DNA by different 
strands of targeting DNA at the two ends, as shown in Fig. 1a.

In the course of our studies, we identified a notable difference 
in hDNA formation between the two flanking regions, with a 
greater level of hDNA detected in the upstream, promoter- 
proximal flanking region (Fig. 3) at both TRP1 and MET17. The 
differences in frequency of hDNA formation between the 
upstream and downstream loci could be due to differential end 
processing by nucleases or to different lengths of DNA engaged 
in hDNA during strand invasion. Alternatively, it is possible that 
hDNA forms efficiently at one or both ends of the targeting 
fragment but that the positions of strand cleavages required to 
connect both strands of the targeting DNA with the chromo- 
somal locus vary in the upstream and downstream regions, 
resulting in the difference in frequency of retention of hDNA in 
the resulting transformants. To gain insight into the factors that 
influence formation of gene targeting intermediates, we first 
considered the possibility that some aspect of transcription 
initiation or transcription-related chromatin remodeling led to 
higher hDNA formation in the promoter-proximal flanking 
region. The stimulatory effects of transcription on recombinase-
tion in general (24, 25) and on gene targeting in particular (26) have been appreciated for some time, suggesting transcription could be responsible for the observed disparity in hDNA formation. To examine this question, we inverted the met17::ADE2 locus on chromosome XII so that it is transcribed toward the centromere and repeated the transformation experiments. If the difference in hDNA in the upstream and downstream flanking regions was attributable to transcription alone, we predicted that the disparity would be retained when targeting the inverted locus, but in fact the difference disappeared (Fig. 3b) even though the inverted locus was still transcribed at 60% of the level of the noninverted locus. This result makes it extremely unlikely that transcription is responsible for the disparity observed when targeting the original met17::ADE2 locus.

A second possibility is that the disparity has to do with the effects of replication on gene targeting. A recent paper showed a dramatic effect of replication on the frequency of gene targeting in Schizosaccharomyces pombe, but the effect was limited to particular flanking sequences overlapping an active origin of replication (27). To test the effects of replication on hDNA formation during gene targeting, we mutated the ARS1 origin at the TRP1 locus on both the targeting DNA and on the chromosomal trp1::URA3 allele. hDNA formation at the downstream locus, in the vicinity of the ars1 mutation, was completely unaffected. Surprisingly, however, hDNA was reduced at the upstream locus (Fig. 3a). This result shows that hDNA formation at TRP1 is affected by the presence of the active replication origin. It is unclear whether the effect comes from binding of origin-specific proteins or from active initiation of DNA replication. We favor the latter hypothesis based on the fact that hDNA formation in the vicinity of ARS1, where origin-specific proteins would bind, was unaffected by the ars1 mutation.

Taken together, our studies lead to the firm conclusion that the early steps in gene targeting in wild-type yeast are analogous to those in DSBR, with the two ends of the targeting DNA separately engaging their homology regions in the chromosome as if each were one side of two separate double-strand breaks (Fig. 1a). From this point on, the analogy to DSBR breaks down because the opposite sides of the breaks are absent, and the only recombination/repair gene so far shown to be absolutely required for gene targeting in yeast is RAD52. The requirement for RAD52 in gene targeting in vertebrate cells is less strict (28, 29), although a defect in gene targeting is one of the few phenotypes of vertebrate RAD52−/− mutants (28, 29). Interestingly, gene targeting in mammalian cells is absolutely dependent on Ercc1 (30), which together with Xpf codes for a heterodimeric endonuclease. The requirement for the Ercc1 homolog in yeast, RAD10, is less stringent, although targeting frequencies are reduced 3- to 40-fold in rad10 and rad1 (Xpf homolog) mutants (10, 31–34). The peculiar genetic requirements of gene targeting are reminiscent of those for the single-strand annealing pathway for direct repeat recombination (35, 36), which suggests that a similar intermediate exists during gene targeting that requires the mediator and/or annealing functions of Rad52 to form and the endonuclease function of Rad1/10 to be processed. Although the details of such a mechanism remain unknown, it is clear from the changes in hDNA observed at the inverted MET17 locus and in the ars1 mutant at TRP1 that the formation and resolution of these intermediates are influenced a great deal by the chromosomal microenvironment of the targeted locus.

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