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Strand Bias in Targeted Gene Repair Is Influenced by Transcriptional Activity

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Modified single-stranded DNA oligonucleotides can direct nucleotide exchange in *Saccharomyces cerevisiae*. Point and frameshift mutations are corrected in a reaction catalyzed by cellular enzymes involved in various DNA repair processes. The present model centers on the annealing of the vector to one strand of the helix, followed by the correction of the designated base. The choice of which strand to target is a reaction parameter that can be controlled, so here we investigate the properties of strand bias in targeted gene repair. An in vivo system has been established in which a plasmid containing an actively transcribed, but mutated, hygromycin-enhanced green fluorescent protein fusion gene is targeted for repair and upon conversion will confer hygromycin resistance on the cell. Overall transcriptional activity has a positive influence on the reaction, elevating the frequency. If the targeting vector is synthesized so that it directs nucleotide repair on the nontranscribed strand, the level of gene repair is higher than if the template strand is targeted. We provide data showing that the targeting vector can be displaced from the template strand by an active T7 phage RNA polymerase. The strand bias is not influenced by which strand serves as the leading or lagging strand during DNA synthesis. These results may provide an explanation for the enhancement of gene repair observed when the nontemplate strand is targeted.

The use of oligonucleotides to direct single-base changes in DNA has become an important strategy for understanding gene function. While processes for disabling targeted genes and creating knockout strains have provided an enormous amount of information, it is now becoming evident that subtle changes in the genomes of many organisms constitute a centerpiece of genetic variability. Specifically, as information regarding the human genome accumulates, the importance of single-nucleotide polymorphisms is now clear. These simple alterations, often causing no visible phenotype variation, may hold the key to the spectrum of responses to many therapeutic agents. In recognition of the importance of single-nucleotide polymorphisms, several technologies have emerged that aim to create animal and plant models that will recapitulate the nucleotide alterations without disrupting the integrity of the whole gene. Many of these technologies employ synthetic oligonucleotides as vectors for directing the introduction of the specific changes.

Our laboratory has developed a series of these oligonucleotide vectors that can create single-base changes in chromosomal and episomal targets. One of these vectors is the chimeric RNA/DNA oligonucleotide (chimera), which consists of cRNA and DNA residues folded into a double hairpin conformation. Chimeras have been shown to direct base replacement or base insertion in yeast (21, 27), mammalian cells in culture (1, 6, 15, 38), animal models (2, 14, 16, 26, 31), and plants (3, 28, 40). While the mechanism of targeted gene repair is not fully elucidated, a number of important steps have been established (5, 9, 10), including the identification of the DNA strand of the chimera as the active repair component (10) and the observation that the central reaction intermediate is a double D-loop joint molecule (9). The former observation came from a systematic study in which the chimera was broken down into its elemental pieces in order to identify functional domains. By extending earlier work by Sherman and colleagues (23, 36, 37), we found that the DNA molecule itself was active in gene repair reactions. Recently we demonstrated that single-base mutations can be corrected by single-stranded oligonucleotides modified at each terminus by a unique number of phosphorothioate linkages (10). These modifications confer resistance to degradation by nucleases that are present in most eukaryotic cells and act to reduce vector activity. These modified single-stranded vectors have now been used to repair both point and frameshift mutations in *Saccharomyces cerevisiae* (21) and HeLa cells (25) at a higher frequency than the intact chimera. The simplicity of design and synthesis makes single-stranded vectors more attractive candidates for functional genomics applications than their double-stranded counterparts.

While carrying out those initial experiments with the single-stranded vectors, we noticed that repair occurred at a higher frequency if the vectors were designed to hybridize to the nontemplate (nontranscribed) strand of the target gene (21). These results reinforce the pioneering work of Sherman and colleagues (37), in which strand bias repair activity in yeast was initially demonstrated. In the earlier work, however, high levels of oligonucleotide were required, presumably to overcome the degradation of the vector by cellular nucleases. The observation that single-stranded oligonucleotides exhibit a definite strand bias for targeted gene

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repair is intriguing, and it is an important fact when considering optimal vector design.

This observation could be explained by a sequence specificity of the molecule or a steric hindrance that precludes the binding of the vector. But, while these and other explanations are possible, two hypotheses seem most plausible and are readily testable. Since the RNA polymerase transits the transcribed (T) strand, its movement, or the presence of RNA itself, may dislodge and/or displace the oligonucleotide positioned to direct nucleotide repair. This type of disruption may occur prior to the completion or partial completion of any repair events. Concurrently, the RNA polymerase may alter the likelihood of complex formation between the vector and the target in a strand-specific manner. Alternatively, DNA replication may determine the accessibility of the lagging or leading strand to targeting vectors (22). To test both hypotheses, we conducted a series of experiments in which strand bias in gene repair was investigated within the context of a transcriptionally repressed or activated gene or as a function of DNA replication. We observe a higher level of gene repair on the transcribed strand when the gene is transcriptionally active. In related assays, the effect of RNA polymerase activity on the stability of preformed complexes was examined in vitro. Little detectable effect is seen as a function of which strand serves as the leading or lagging strand during DNA synthesis. Our results suggest that the process of transcription, in fact, can dislodge a single-stranded DNA vector bound to the template strand. These observations provide support for the hypothesis that strand preference in gene repair reactions is dictated, at least in eukaryotes, by the transcriptional status of the template strand.

MATERIALS AND METHODS

Plasmids. The plasmid pAURHyg(del)eGFP was used as the target and has been described previously (21). The plasmid pYesHyg(del)eGFP was constructed by inserting the hygromycin gene fused to enhanced green fluorescent protein (eGFP) into pYes2/CT (Invitrogen, Carlsbad, Calif.) between the *Kpn*I and *Not*I restriction sites. First, the hygromycin-eGFP cassette was excised from pAURHyg(del)eGFP plasmids at the *Kpn*I and *Not*I sites and was then ligated into pYes2/CT plasmid. Thus, the pYesHyg(del)eGFP plasmids have the same mutation as pAURHyg-(del)eGFP (a one-base deletion at nucleotide position 137). Plasmid pGemHyg-(del)eGFP was constructed by inserting the hygromycin-eGFP cassette (21) from pAURHyg(del)eGFP at the *Kpn*I and Sa1I restriction sites into pGem-blue (Promega). The plasmid pAURHyg(del)eGFP-R is identical to the plasmid pAURHyg-(del)eGFP, except that the direction of the fusion gene fragment is inverted. This reversal was accomplished as follows: the plasmid pAUR123 was cut with *Bam*HI, releasing the promoter fragment, PADH1-TADH1. This fragment was purified, religated back into pAUR123, and then screened for the reversed orientation by colony PCR with forward primers (pHyg5851F: 5′ AGTCCGATCAGCTCATA AAG and pAUR123F 5′ TCTGCACAATATTTCAAGC). Only plasmids containing a promoter in the opposite orientation would be amplified with these two forward primers. Then the Hyg(del)eGFP cassette with *Kpn*I and *Sal*I sites was inserted into pAU123-R. To expand the plasmid and assure that the replication polarity of the strands was reversed, a 2.3-kb fragment was also inserted in the ampicillin resistance gene, creating the plasmid pAURHyg(del)eGFP-R.

Oligonucleotides. Hyg3S/74T, Hyg3S/74NT, and Kan3S/70T are single-stranded oligonucleotides containing three phosphorothioate linkages at 3′ and 5′ termini with lengths of 74 and 70 nt, respectively. Vector Hyg3S/74T was designed to target the transcribed strand of hygromycin gene, while Hyg3S/74NT targets the nontranscribed strand. Kan3S/70T is a nonspecific oligonucleotide having no complementarity to the hygromycin target gene.

Electroporation of *S. cerevisiae*. Five micrograms of plasmid DNA were transfected into *S. cerevisiae* LSY678 (*MAT***a** *leu2-3,112 trp1-1 ura3-1 his3-11,15 ade2-1 can100*) (a gift from L. Symington, Columbia University) by electroporation. Briefly, cells were grown at 30°C to a density of approximately 10^8 cells/ml in 40 ml of yeast-peptone-dextrose (YPD) medium, washed twice with 25 ml of sterile H_2O and once with 1 ml of 1 M sorbitol, and then resuspended in 120 μl of 1 M sorbitol. Aliquots (40 μl, 10^8 cells) were incubated with 5 μg of DNA on ice for 5 min, transferred to a 0.2-cm cuvette, and electroporated using a BIO-RAD Gene Pulser apparatus (Richmond, Calif.) (1.5 kV, 25 μF, 200 Ω, 1 pulse, 5 s per pulse-length). Cells were immediately resuspended in 1 ml of YPD supplemented with 1 M sorbitol and shaken at 30°C at a speed of 300 rpm for 6 h. Two hundred microliters of yeast cells were spread on YPD-aureobasidin A (0.5 μg/ml) selective plates or minimal Sabouraud dextrose base Ura-lacking (Ura$^-$) selective plates and cultured at 30°C for 3 days.

Episomal targeting of mutated hygromycin in LSY678 containing pAURHyg(del)eGFP plasmids. Five micrograms of Hyg3S/74T or Hyg3S/74NT were electroporated into *S. cerevisiae* LSY678 containing pAURHyg(del)eGFP by the same protocol as mentioned above, except that for each sample, 200 μl of yeast cells was spread on YPD-hygromycin (300 μg/ml) plates and another 200 μl of 10^5 diluted yeast cells was spread on YPD-aureobasidin A (0.5 μg/ml) plates simultaneously.

Episomal targeting of mutated hygromycin in LSY678 containing pYesHyg(del)eGFP plasmids. LSY678 cells containing pYesHyg(del)eGFP were grown in 10 ml of SUR media (0.67% nitrogen base, 2% raffinose, 0.077% Ura− supplements) for 16 h and then adjusted to an optical density at 600 nm of 0.2 to 0.3 in 40 ml of SUR media; the culture was continued until the cell density reached 2×10^7 cells/ml. The cells were pelleted at $3,000 \times g$ for 5 min and then resuspended in 1 ml of SUR medium supplemented with 25 mM dithiothreitol and shaken at 300 rpm for 20 min. The cells were then washed twice with 25 ml of sterile H_2O and once with 1 ml of 1 M sorbitol and resuspended in 1 M sorbitol (120 μl). Aliquots of 40 μl of yeast suspension were electroporated with 5 μg of Hyg3S/74T or Hyg3S/74NT using a BIO-RAD Gene Pulser apparatus (1.5 kV, 25 μF, 200 Ω, 1 pulse, 5 s/pulse). Cells were immediately resuspended in 3 ml of SUDex (0.67% nitrogen base, 2% dextrose, 0.077% Ura- supplements) or SUG (0.67% nitrogen base, 2% galactose, 0.077% Ura− supplements) medium, respectively, supplemented with 1 M sorbitol, and shaken at 300 rpm for 16 h. Two hundred microliters of 10^5 diluted yeast cells was spread on SURH600 (0.67% nitrogen base, 2% raffinose, 0.077% Ura− supplements, 0.15% agar [pH 6.5], hygromycin [600 μg/ml]) plates and cultured at 30°C for 3 days.

Corrected plasmid rescue and DNA sequencing. Correction of the hygromycin target at nucleotide 137 was confirmed by DNA sequencing. Colonies were picked from a hygromycin plate and grown in 5 ml of YPD at 30°C overnight. Cells were collected by centrifugation, treated with 0.2 ml of buffer A (2% Triton X-100, 1% sodium dodecyl sulfate (SDS), 100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]), 0.2 ml phenol-chloroform (25:24), and 0.2 ml of glass beads (Sigma), and then vortexed for 2 min, spun down, and ethanol precipitated, and the DNA was dissolved in H_2O. Five microliters of resuspended plasmid was transformed into 20 μl of competent bacteria (*Escherichia coli* DH10B) by a Cell Porator Apparatus (Life Technologies) as described by the manufacturer. Each mixture was transferred to a 1-ml SOC culture (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) and incubated at 37°C for 1 h; 200 μl of oligonucleotides was spread on ampicillin (100 mg/ml) plates and cultured at 37°C overnight. Colonies were picked and grown overnight in 5 ml of Luria-Bertani broth at 37°C. Plasmid DNA was isolated by Concert rapid plasmid miniprep kit (Life Technologies) with 5 μl of a miniprep product used as a template for Sanger dideoxy sequencing with the ABI prism kit on an automated ABI 310 capillary sequencer.

D-loop formation. Formation of joint molecules was carried out in two steps. A 15-min preincubation with 5 nM ^{32}P-74-mer oligonucleotide and 1 μM *E. coli* RecA protein in a solution containing 1 mM γ-S-ATP, 25 mM Tris-OAc (pH 7.5), 1 mM $Mg(OAc)_2$, and 1 mM dithiothreitol was carried out at 37°C. Strand exchange was then conducted in the presence of 20 nM pGemHyg(del)eGFP plasmid and 10 mM $MG(OAc)_2$ for 4 min at 37°C (molar ratio of plasmid to 74-mer of 4:1). The joint molecules were deproteinated by the addition of 1% SDS at 4°C. SDS and associated protein were removed by adding KCl to a 100 mM concentration and spinning the solution at 8,000 rpm for 5 min at 4°C. A buffer exchange into 10 mM Tris, 1 mM EDTA was effected using a Centri-Spin 20 (Princeton Separations) or ChromaSpin 400 (Clontech Laboratories, Inc.) spin column. Free oligonucleotide was removed from solution using the ChromaSpin 400 column. The presence of joint molecules was confirmed by agarose gel electrophoresis prior to use in the in vitro transcription experiments.

Transcription. In vitro transcription experiments were carried out using T7 or Sp6 RNA polymerase transcription kits (Ambion, Austin, Tex.). Briefly, the Hyg3S/74T-plasmid joint molecules or Hyg3S/74NT-plasmid joint molecules were incu-

bated with either T7 or SP6 RNA polymerase at 37°C for 10 min. Then, the samples were incubated with either H_2O or 1 mg of RNase H (Roche)/ml at 37°C for an additional 10 min. Finally, reactions were terminated with 1% SDS. Reaction products were then analyzed by electrophoresis in a 1% agarose gel (1× Tris-borate-EDTA, 90 V, 1.5 h.) Gels were dried on Whatman DE81 filter paper using a Gel Dryer Vacuum System (FisherBioTech) for 2 h at 80°C and visualized by using a Typhoon 8600 PhosphorImager (Molecular Dynamics Inc., Sunnyvale, Calif.).

RESULTS

Single-stranded oligonucleotide vectors were designed to repair a point mutation in the plasmid pAURHyg(del)eGFP (21). This plasmid contains a fusion construct consisting of a hygromycin gene and an eGFP gene regulated by the ADH1 promoter (PADH1). The plasmid also contains an ARS1 element and a CEN element maintaining the plasmid in low copy number. The deletion mutation is located within the hygromycin gene; hence, correction by the vector enables hygromycin and eGFP gene expression. As shown in Fig. 1, the wild-type sequence is TAT at the targeted site, while the mutant sequence contains a deletion (Δ), TAΔ; repair of the mutant sequence by the oligonucleotide regenerates the TAC codon.

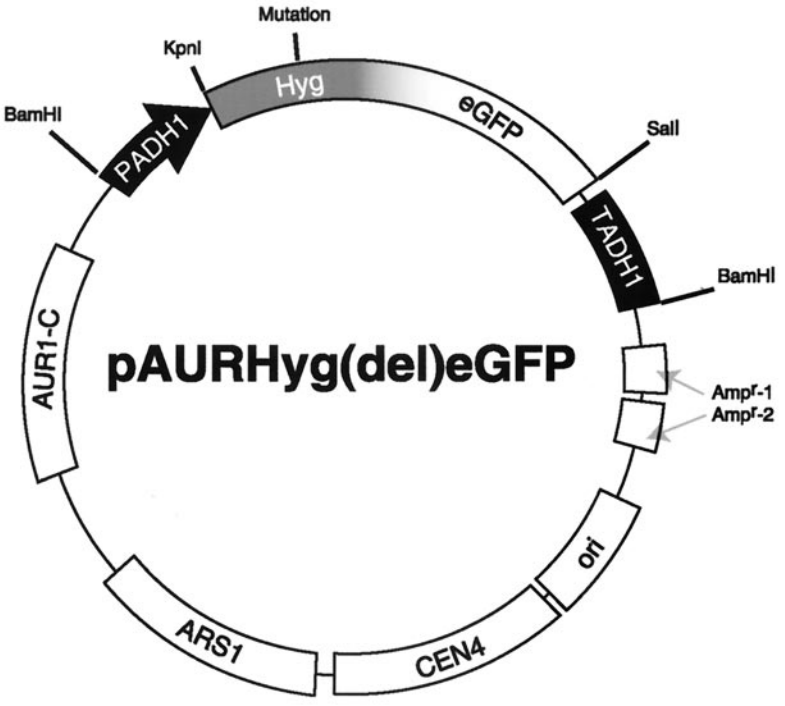
The oligonucleotide vectors are 74 bases in length and are modified by three phosphorothioate linkages located at both the 3′ and 5′ termini. Two vectors are designed to direct the insertion of a C residue recreating the TAC codon. The difference between Hyg3S/74T and Hyg3S/74NT is that the former directs repair on the template (T) strand, while the latter directs repair on the nontemplate (NT) strand. Kan3S/70T is a 70-mer that lacks complementarity to the target site.

The plasmid pAURHyg(del)eGFP was established as a stable episome in yeast strain LSY678 (see Materials and Methods) being maintained at a low copy number. Vectors Hyg3S/74T, HygS/74NT, and Kan3S/70NT were electroporated separately into LSY678, and more than 80% of the cells were observed to be transformed (data not shown). Repair of the frameshift mutation was measured by growing the transformed cells under hygromycin selection. The efficiency of the repair of the mutated fusion gene is determined by dividing the number of hygromycin-resistant colonies by the number of aureobasidin-resistant colonies. The latter count is used to normalize transfection efficiency. As shown in Fig. 2, top, Hyg3S/74NT directed a higher level of mutation repair, consistent with earlier results (21). The nonspecific vector, Kans.3S/70T, produced no detectable repair events. In addition, cells were isolated from a hygromycin-resistant colony and examined under a confocal microscope (Fig. 2, bottom). The picture displays yeast cells expressing green fluorescent protein, confirming at the phenotypic level that gene repair has occurred. Subsequent DNA sequence analyses also confirmed that the targeted nucleotide was altered specifically (data not shown).

The strand bias for gene repair exhibited by the targeting vectors Hyg3S/74NT and Hyg3S/74T may result from the direction of replication on the plasmid target. Ellis et al. proposed that a correlation exists between oligonucleotide annealing and the strand of the target serving as the lagging strand in DNA replication (8). Apparently, these authors envision the lagging strand being more available for vector binding or hybridization than the more continuously replicated leading strand. This hypothesis can be tested directly in our system by inverting the Hyg-eGFP cassette and creating the plasmid pAURHyg(del)eGFP-R (Fig. 3A). The plasmid is identical to pAURHyg(del)eGFP, except that the orientations of the targeted fusion gene and the promoter are reversed. While the template strand and nontemplate strand remain intact, the lagging and leading strands are switched in this construct. Hence, a result in which Hyg3S/74T was more active than Hyg3S/74NT in directing gene repair would indicate that the lagging or leading strand influenced which strand was more amenable to correction. If Hyg3S/74NT remained the more active of the two vectors, then transcriptional activity is likely the determining factor in strand bias. As shown in Fig. 3B, Hyg3S/74NT was found to remain more active than Hyg3S/74T in directing gene repair in pAURHyg(del)eGFP-R. In other words, the nontemplate (nontranscribed) strand, which remains the same in either orientation, continues to be more amenable to gene repair at each dosage tested. While this does not exclude replication as a major factor in determining the strand bias effect, strand polarity of DNA replication may not be the most dominant parameter in controlling strand selectivity of gene repair.

To this end, we examined the activity of the RNA polymerase itself and/or the production of mRNA as a potential factor in determining strand bias. Transcription could enable binding of Hyg3S/74NT to the nontemplate strand due to the increased accessibility of the plasmid target, or it could displace Hyg3S/74T from the template strand. To test this hypothesis, the plasmid pYesHyg(del)eGFP was created; it is depicted in Fig. 4A. The parent plasmid (pYes2) is a vector in which the expression of the inserted gene (here, the fusion construct hygromycin-eGFP) is under the control of the Gal1 promoter. Growth in the presence of galactose as the sole carbon source induces expression of the inserted template, while the use of dextrose represses gene expression. The modified plasmid, pYes/Hyg(del)eGFP, is constructed so that the same DNA strand, transcribed in pAURHygeGFP, serves as the template in the pYes2 variant plasmid. Hence, Hyg3S/74T and Hyg3S/74NT will retain the same relationships to the template and nontemplate strands described previously.

The modified plasmid was introduced into LSY678, and the strain was grown in the presence of dextrose or galactose. Yeast cells were transformed by electroporation with either of the oligonucleotide vectors depicted in Fig. 1. As displayed in Fig. 4B, the nonspecific vector produced no resistant colonies. Cells grown in the presence of dextrose and treated with Hyg3S/74T produced a low correction efficiency, while transformation with the 74NT vector exhibited a level of enhancement similar to that reported previously (21). When cells grown in the presence of galactose were transformed with either Hyg3S/74T or Hyg3S/74NT, an overall increase in correction efficiency was observed for both vectors. But a substantial increase in colony count was apparent when Hyg3S/74NT was compared to Hyg3S/74T, and DNA sequence analyses confirmed the precision of the targeted repair (Fig. 4C). Taken together, these results indicate that if a gene contained in this plasmid is transcriptionally active, it appears to be more amenable to targeted gene repair, and further, targeting of the nontemplate strand under these conditions enables an even higher level of activity. This effect is unique to modified single-stranded vectors because while double-stranded chimeric RNA/DNA oligonucleotides can direct conversion at a low



wild type (T) GATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGC
(NT) CTACATCCTCCCGCACCTATACAGGACGCCCATTTATCGACG
D V G G R G Y V L R V N S C

deletion GATGTAGGAGGGCGTGGATAΔGTCCTGCGGGTAAATAGCTGC
CTACATCCTCCCGCACCTATCCAGGACGCCCATTTATCGACG
D V G G R G ●

Hyg3S/74T 5'- G*T*A*GAAACCATCGGCGCAGCTATTTACCCGCAGGACGT
ATCCACGCCCTCCTACATCGAAGCTGAAAGCAC*G*A*G-3'

Hyg3S/74NT 5'- C*T*C*GTGCTTTCAGCTTCGATGTAGGAGGGCGTGG
ATACGTCCTGCGGGTAAATAGCTGCGCCGATGGTT
TC*T*A*C -3'

Kan3S/70T 5'-C*A*T*CAGAGCAGCCAATTGTCTGTTGTGCC
CAGTCGTAGCCGAATAGCCTCTCCACCCAAAC
GGCCGG*A*G*A-3'

FIG. 1. A model system for gene repair utilizing the plasmid pAURHyg(del)eGFP. The plasmid, pAURHygeGFP, has a synthetic expression cassette which contains a hygromycin B/eGFP fusion gene controlled by the alcohol dehydrogenase (ADH1) constitutive promoter. The plasmid pAURHyg(del)eGFP (depicted) contains a one-base deletion mutation at nucleotide 137 within the hygromycin B coding sequence, which makes a frameshift mutation of TAT (tyrosine) to TAG (stop codon, symbolized as ● in-frame). Correction of pAURHyg(del)eGFP is achieved by the insertion of a C residue at nucleotide 137. Hyg3s/74T and Hyg3s/74NT are single-stranded oligonucleotides containing 74 nucleotides; T refers to an oligonucleotide designed to target the transcribed strand, while NT refers to an oligonucleotide designed to target the nontranscribed strand. Kan3S/70T is a nonspecific molecule with the length of 70 nucleotides bearing no sequence homology to the target. The asterisk refers to phosphorothioated linkages within the oligonucleotide, located at both 5′ and 3′ termini. S, sense strand.

Target	Vector (μg)	Hyg^r (10^5)	Aur^r (10^5)	Corr. Eff. (10^5)
pAURHyg(del)eGFP	0	0	99	0.0
pAURHyg(del)eGFP	Hyg3S/74T (1.0)	1	50	0.02
pAURHyg(del)eGFP	Hyg3S/74T (2.5)	98	94	1.04
pAURHyg(del)eGFP	Hyg3S/74T (5.0)	317	94	3.37
pAURHyg(del)eGFP	Hyg3S/74T (10.0)	170	88	1.93
pAURHyg(del)eGFP	Hyg3S/74NT (1.0)	14	73	0.19
pAURHyg(del)eGFP	Hyg3S/74NT (2.5)	513	83	6.18
pAURHyg(del)eGFP	Hyg3S/74NT (5.0)	1807	91	19.85
pAURHyg(del)eGFP	Hyg3S/74NT (10.0)	1405	132	10.65
pAURHyg(del)eGFP	Kan3S/70NT (5.0)	0	76	0.0

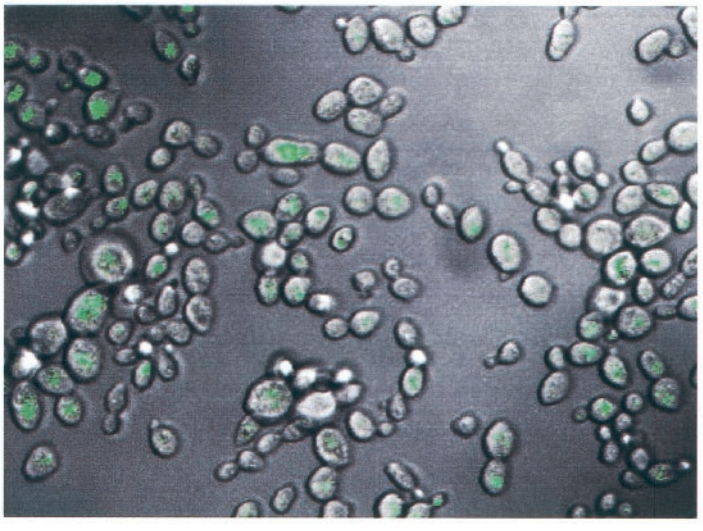
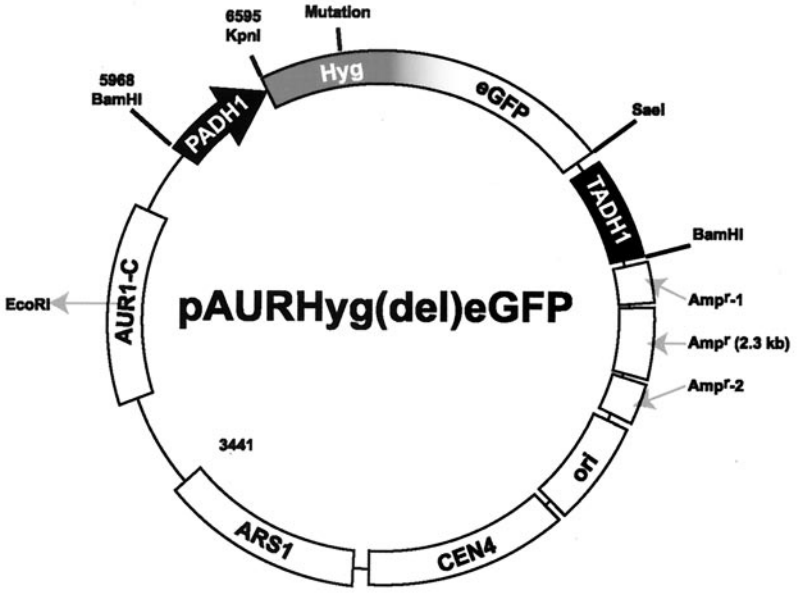


FIG. 2. Dose response and green fluorescence protein (GFP) expression. (Top) Strand bias of gene repair. Dose response of gene repair was established using Hyg3S/74NT, Hyg3S/74T, or Kan3S/70T. Different amounts of vector (0, 1.0, 2.5, and 5.0 μg) were electroporated into *S. cerevisiae* LSY678 containing pAURHyg(del)eGFP. After a recovery of 6 h in 1 ml of YPD supplemented with 1 M sorbitol, 200 μl of yeast cells were spread on hygromycin-selective YPD agar plates and cultured at 30°C for 3 days. The average numbers of hygromycin-resistant and aureobasidin-resistant colonies per 10^5 targeted cells are given. Correction efficiency (Corr. Eff.) reflects the number of hygromycin-resistant colonies divided by the number of aureobasidin-resistant colonies, with the latter number being used to normalize electroporation variability. The numbers are an average from five experiments with a variance of ±10%. (Bottom) Hyg3S/T4NT (5 μg). Single hygromycin-resistant colonies were picked and cultured in YPD media for 1 to 3 h as described in Materials and Methods. The expression of GFP was visualized with a Zeiss LSM 510 confocal microscope using a 505- to 550-nm band-pass (see reference 2 for details).

level, they exhibit no strand bias in the targeted repair activity (data not shown).

The observations reported above support the notion that the nontranscribed strand is a more productive target for the vector. One explanation for this difference is that the vector is displaced by the movement of RNA polymerase through the transcribed region. To test the possibility of vector displacement, a new construct was created (Fig. 5A). The plasmid pGem/Hyg(del)eGFP was produced by inserting the Hyg(del)eGFP cassette from pAURHyg(del)eGFP into a pGem-blue expression construct, where the expression of the gene is under the control of the T7 promoter in one direction and the Sp6 promoter in the other. Hence, addition of T7 RNA polymerase initiates transcription of the Hyg(del)eGFP T strand, while the Hyg(del)eGFP NT strand is transcribed by Sp6 polymerase. Therefore, the potential displacement of

A.

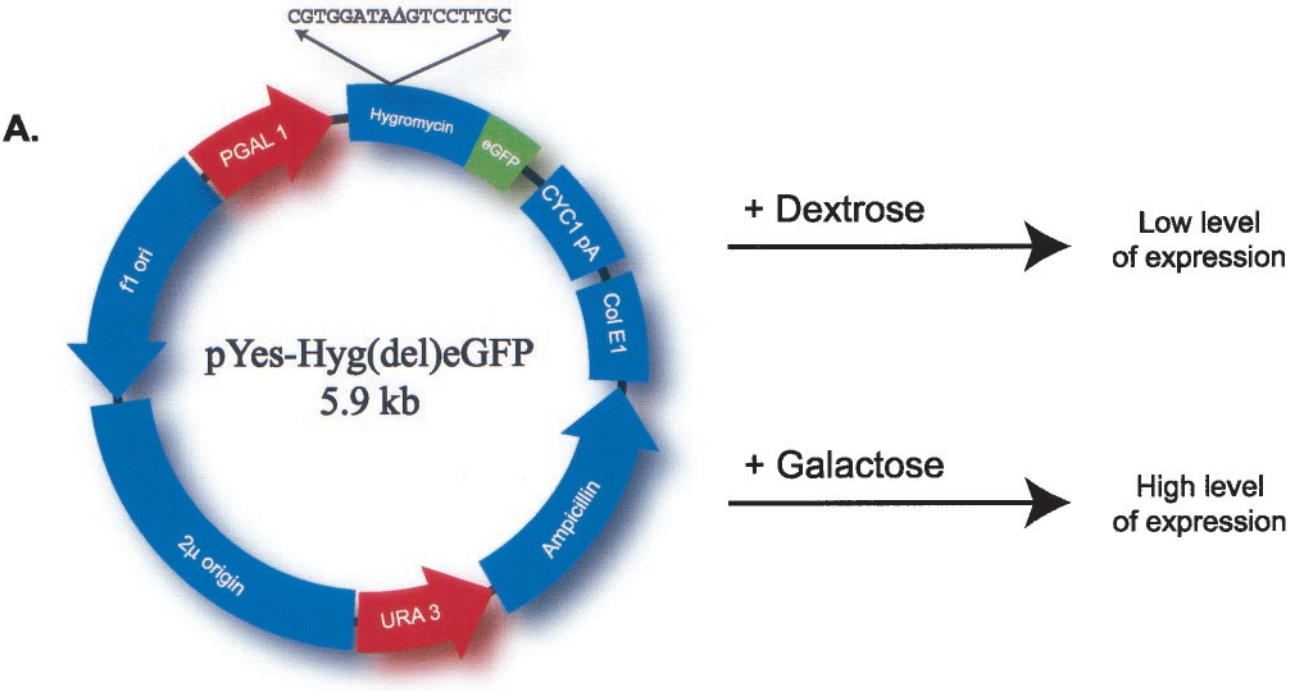


B.

Target	Oligo Vector (μg)	Hygr (10^5)	Corr. Eff. (10^5)
pAURHyg(del)eGFP-R	-	0	0.0
pAURHyg(del)eGFP-R	Hyg3S/74T (1)	3	0.03
pAURHyg(del)eGFP-R	Hyg3S/74T (2.5)	139	1.47
pAURHyg(del)eGFP-R	Hyg3S/74T (5)	354	3.86
pAURHyg(del)eGFP-R	Hyg3S/74NT (1)	23	0.24
pAURHyg(del)eGFP-R	Hyg3S/74NT (2.5)	1490	5.25
pAURHyg(del)eGFP-R	Hyg3S/74NT (5.0)	1639	18.2
pAURHyg(del)eGFP-R	Kan3S/70T (5.0)	0	0.0

FIG. 3. Inversion of the fusion gene with the plasmid target. (A) Plasmid pAURHyg(del)eGFP-R contains the same elements found in pAURHyg(del)eGFP, except that the fusion gene is inverted. This construction reverses the leading and lagging strands during DNA replication but maintains the same strand identity (NT and T) for transcription. (B) Plasmid pAURHyg(del)eGFP-R was stably maintained in LSY678 by aureobasidin selection, and the mutant hygromycin-eGFP fusion gene was targeted with either Hyg3S/74T or Hyg3S/74NT as indicated. The average number of hygromycin-resistant colonies per 10^5 targeted cells is presented for each dosage, as well as for the nonspecific vector Kan3S/70T. Correction efficiency (Corr. Eff.) is calculated by dividing the number of hygromycin-resistant colonies by the number of aureobasidin-resistant colonies to normalize for electroporation variability. Variation in colony number was ±10%.

Hyg3S/74T by transcriptional activity can be tested using this construct. The experiment was begun by creating a D-loop joint molecule consisting of pGem/Hyg(del)eGFP and either Hyg3S/74T or Hyg3S/74NT through the action of RecA protein. RecA protein catalyzes D-loop formation by introducing the radiolabeled oligonucleotide into the plasmid at the site of complementarity; joint molecules were isolated by gel filtration (33) and visualized by gel electrophoresis. As shown in Fig. 5B, ^{32}P-labeled Hyg3S/74NT and Hyg3S/74T are taken up by pGem/Hyg(del)eGFP to form a stable D-loop structure (lanes 2 and 8, respectively). Addition of T7 RNA polymerase to a reaction mixture containing the 74NT D-loop did not result in displacement of the ^{32}P-labeled oligonucleotide (lane 3). Lane 4 represents a reaction mixture identical to that shown in lane 3 except that no ribonucleoside 5′-triphosphates (rNTPs) are included. Addition of Sp6 RNA polymerase and rNTPs to the 74NT-containing D-loop structure results in oligonucleotide displacement (lane 5). No displacement is observed if rNTPs



B.

Single-Stranded Vector	Hyg(del)eGFP Target	Correction Efficiency (10^5) Dextrose	Galactose
Kan3S/70T	-	0	0
Hyg3S/74T	template strand	0.26	0.91
Hyg3S/74NT	nontemplate strand	0.33	7.33

FIG. 4. Gene repair on transcriptionally activated or repressed templates. (A) The plasmid pYesHyg(del)eGFP includes an inducible promoter (pGal1) and a selective marker (*URA3*), as well as the Hyg(del)eGFP cassette containing the same type of mutation as that in the plasmid pAURHyg(del)eGFP. A high level of transcription from the yeast Gal1 promoter is induced by galactose and repressed by dextrose. (B) *S. cerevisiae* LSY678 containing pYesHyg(del)eGFP plasmids was targeted with Hyg3S/74T, Hyg3S/74NT, or Kan3S/70T and then recovered in either dextrose or galactose culture media overnight. In dextrose media, the average colony number was six when targeting with Hyg3S/74T (a correction efficiency of 0.26 per 10^5 targeted cells) and 20 when targeting with Hyg3S/74NT (correction efficiency of 0.33 per 10^5 targeted cells). However, in media containing galactose, the average number of colonies was 20 with Hyg3S/74T targeting (correction efficiency of 0.91 per 10^5 targeted cells) and 157 with Hyg3S/74NT target (correction efficiency of 7.33 per 10^5 targeted cells) ± 15%. (C) Sequence confirmation of hygromycin gene correction in LSY678 containing pYesHyg(del)eGFP; correction reactions directed by Hyg3S/74NT. Plasmids from single hygromycin-resistant colonies were transformed into *E. coli*, and individual clones were processed for DNA sequence analysis (see Materials and Methods). (Top) LSY678 containing pYesHyg(wt)eGFP displays TAT at codon 46 of the hygromycin B gene. (Middle) LSY678 containing pYesHyg(del)eGFP reveals a deletion at the same position on the hygromycin B gene. (Bottom) A corrected TAC at codon 46 of hygromycin B gene is presented after sequencing of the plasmid targeted by Hyg3S/74NT and isolated from *E. coli*.

are not included in the reaction mix (lane 6). Displacement of ^{32}P-labeled Hyg3S/74T is observed when T7 RNA polymerase and rNTPs are added to a reaction mixture containing a preformed ^{32}P-labeled 74T D-loop (lane 9). No displacement occurs in the absence of rNTPs (lane 10). Finally, addition of Sp6 RNA polymerase does not disrupt the preformed [^{32}P]-labeled 74T D-loop in the presence (lane 11) or absence (lane 12) of rNTPs. SDS was included in the reaction to denature and remove proteins bound to the DNA template noncovalently. Taken together, the data indicate that the activity of RNA

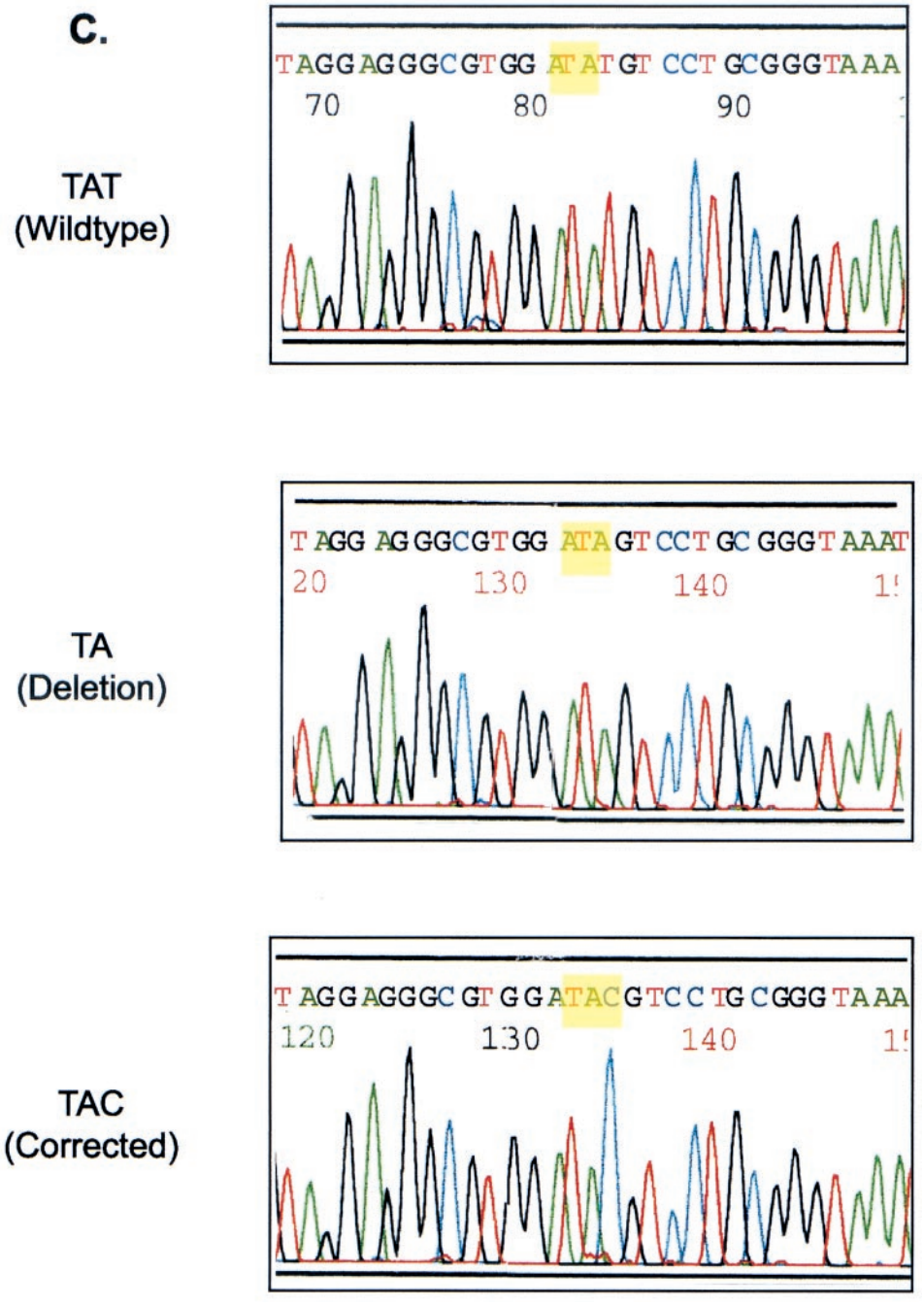


FIG. 4—*Continued*.

polymerase displaces the preformed oligonucleotides Hyg3S/74T and Hyg3S/74NT when the strand to which they are bound is transcribed.

DISCUSSION

Targeted gene repair is a promising strategy for introducing single- or multiple-base changes in specific sites in DNA. While numerous studies have confirmed the validity of this approach, reaction parameters are still being defined. In this work, we examined transcriptional activity as a regulator of frequency. These experiments were undertaken as a result of observations made in a previous study, wherein modified single-stranded vectors designed to correct a point deletion were found to be most effective when targeted to the nontranscribed strand of the gene (21). This difference holds true for replacement and insertion mutations as well.

We confirm that the nontemplate strand is more amenable to gene repair when a deletion in a fusion gene is targeted. By selection for hygromycin resistance and green fluorescence in yeast, we establish that the effect is dose dependent and relies on complementarity to the target site. To test the effect of

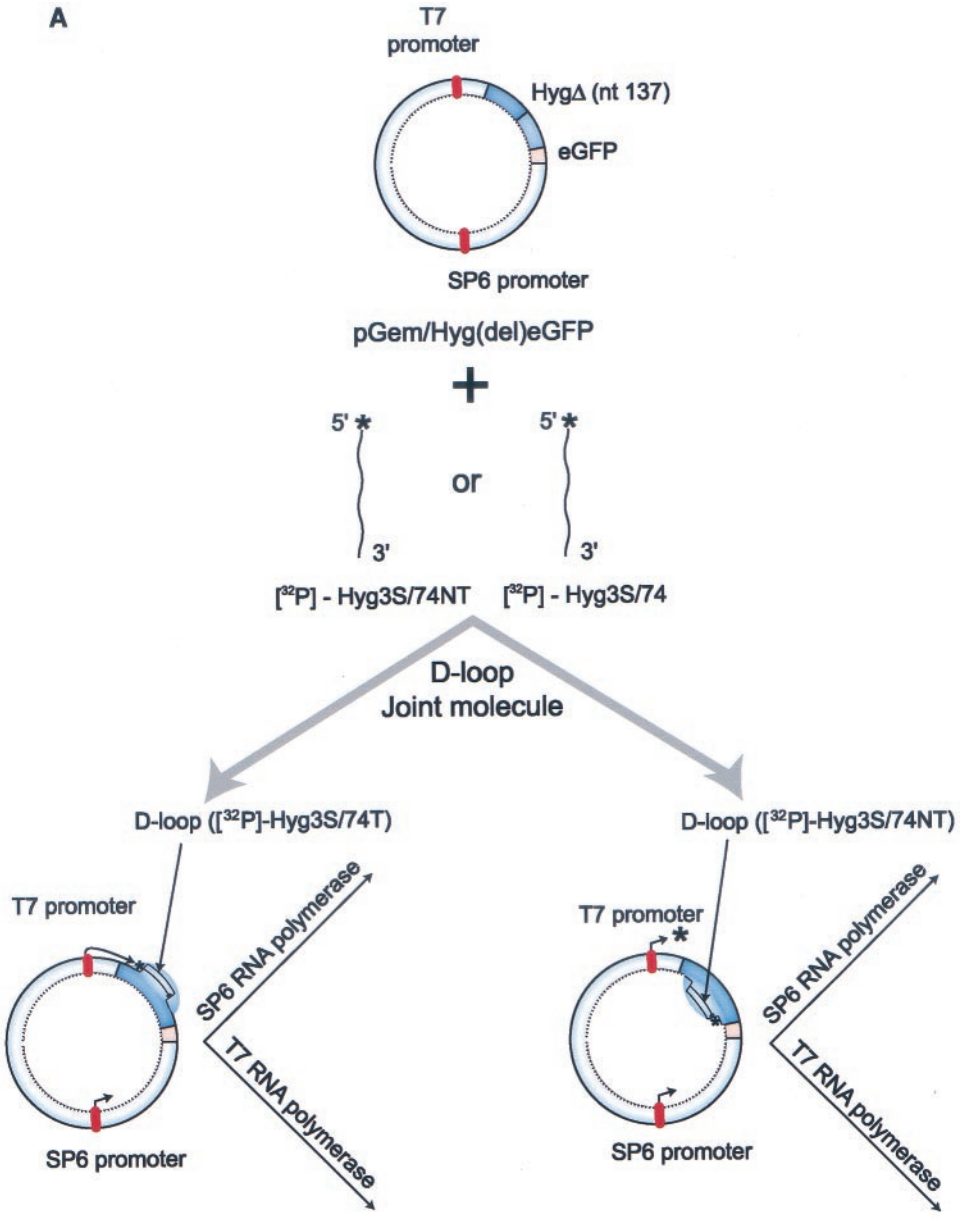


FIG. 5. In vitro transcription influences D-loop stability. (A) Plasmid pGemHyg(del)eGFP was incubated with ^{32}P-labeled oligonucleotides Hyg3S/74NT or Hyg3S/74T in the presence of RecA to form a stable D-loop. Joint molecules were incubated with either Sp6 or T7 RNA polymerase in the presence of rNTPs to initiate transcription from either strand. (B) In this experiment, various components are added as indicated in the heading to the gel. The nomenclature T and NT refer to strands targeted by Hyg3S/74T or Hyg3S/74NT in pAURHyg(del)eGFP and pYesHy(del)eGFP. With pGEMHyg(del)eGFP, SP6 RNA polymerase transcribes the NT strand while T7 RNA polymerase transcribes the T strand. Hyg3S/74NT anneals to the nontranscribed strand of pAURHyg(del)eGFP and pYesHyg(del)eGFP, while Hyg3S/74T anneals to the transcribed strand.

transcriptional activity on gene repair directly, we constructed a plasmid in which fusion gene expression is repressed or induced, dependent on the presence of dextrose (repressed) or galactose (activated). Two observations were made: first, transcriptional activity increases the overall repair frequency, evidenced by the elevation in hygromycin-resistant colonies produced by Hyg3S/74T in the presence of dextrose or galactose (Fig. 4B); second, targeting the nontemplate strand is seven- to eightfold more effective than targeting the template strand when the fusion gene is being transcribed.

Strand bias using oligonucleotides for site-specific mutagenesis in yeast has been reported previously. Earlier work by Yamamoto et al. (36) showed that *cyc1* mutants in *S. cerevisiae* were corrected at a higher frequency when sense oligonucleotides were used. In their terminology, the sense oligonucleotide, which targets the template or transcribed strand, is more effective than their antisense vector, the opposite result from our present study. The difference in efficiency was high, approaching 20-fold, and the bias was maintained even when the transcriptional activity of the gene was lowered to 2% of nor-

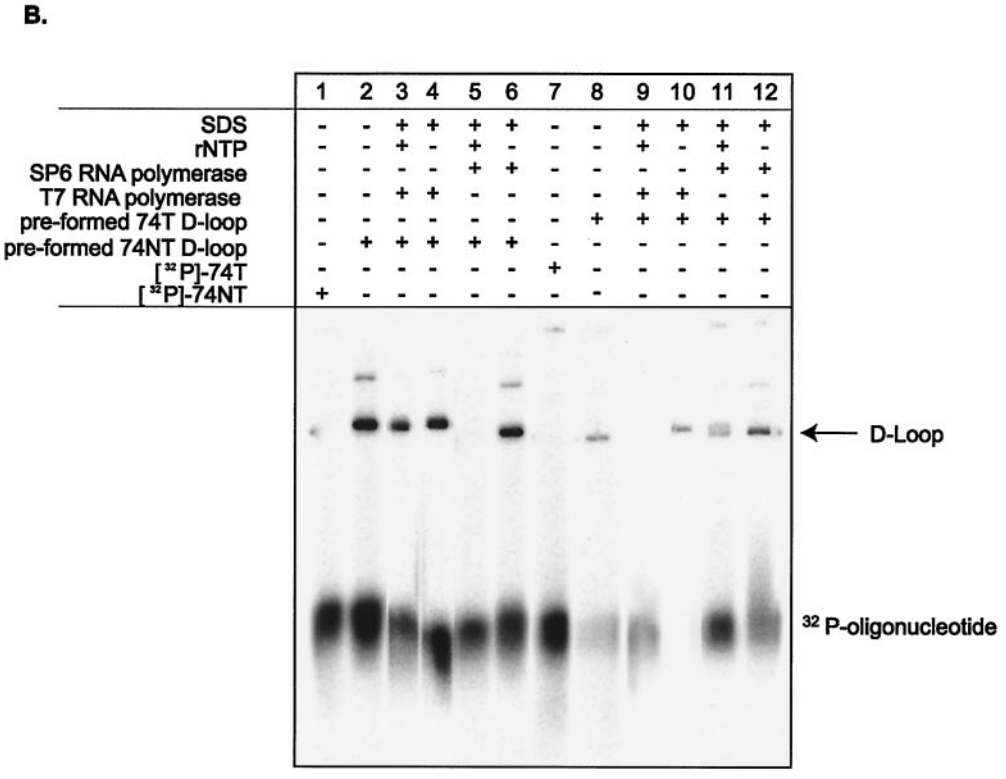


FIG. 5—*Continued*.

mal. The difference between their data and ours may be reconciled in several ways. First, the vectors used in their study were 40 or 50 nt long, while our vectors are 74 nt in length. Second, the two experimental designs were different, particularly in the amount of single-stranded molecules used. To protect against nuclease digestion of the vector, previous experimental protocols, including theirs, employed up to 200 μg (23, 36), while we use 20- to 40-fold less, as our vectors have phosphorothioate-modified linkages connecting the four terminal bases. Modified oligonucleotides are likely to resist nuclease attack and to survive longer in vivo. Finally, the *cyc1* mutation is chromosomal, while our target is extrachromosomal, but preliminary studies have shown that strand bias is also evident at the level of the chromosome (E. Brachman and E. B. Kmiec, unpublished data).

Important links have already been made between transcriptional activity and recombination. Studies have concluded that transcriptionally active DNA fragments are more likely to undergo homologous DNA pairing events (see reference 13 and references therein). Actively transcribed DNA regions were found to participate more in DNA pairing reactions when the region of homology between partners was low (15 to 72 bases). Our results confirm these observations, but the pathway that the Hyg3S/74T or Hyg3S/74NT vectors follow is not fully elucidated. Hence, it may be premature to include targeted gene repair in a scenario that elucidates the effect of transcription on homologous recombination. But what is clear is that the data presented in Fig. 4 support the view that transcriptional activity enhances the overall frequency of targeted gene repair. Our data also suggest that the process of transcription-coupled repair (TCR) is not the major pathway for at least the first correction event. TCR is operational primarily when the lesion is within the template strand (18, 19, 30, 32), but targeted gene repair is most efficient when the vector creates a mismatch through heteroduplex formation with the nontemplate strand (as in a D-loop). TCR reactions occur mainly through nucleotide excision repair (24); studies with yeast (21, 27) and mammalian cells (5, 10) support the notion that two pathways, mismatch repair and homologous pairing, are prerequisites for targeted gene repair. But evidence does exist linking mismatch repair activities with TCR (4, 17, 22); thus, the factors regulating the process of gene repair remain to be fully defined. For example, an elevated number of mutations have been observed to form initially on the nontranscribed strand of the human HPRT gene, a gene transcribed by RNA polymerase II (34, 35). Thus, enzymatic processing events during transcription can actually be focused toward the nontranscribed strand. Since the oligonucleotide may actually be envisioned as a site-specific mutation agent, perhaps some aspects of the directed repair mechanism are part of normal, unrelated, cellular activities.

As we have observed, transcription past a D-loop has different consequences depending upon whether the oligonucleotide is hybridized to the template or nontemplate strand of the plasmid. Release of an oligonucleotide that is hybridized to the template strand implies that transcription can proceed unencumbered through the duplex arm of a D-loop. We propose that RNA polymerase irreversibly displaces the oligonucleotide one base at a time to accommodate RNA synthesis. Resolution of the D-loop is then completed when the template and

nontemplate strands reanneal as usual at the distal end of the transcription bubble. RNA polymerase can also transcribe through most triple-stranded complexes (7, 29, 39), presumably dissociating the oligonucleotide in the process. In D-loops where the oligonucleotide is hybridized to the nontemplate strand of the gene, transcription can proceed without release of the oligonucleotide. Displacement loops formed by the hybridization of peptide nucleic acid to the nontemplate strand of a gene permit transcription read-through, although some site-specific blockage of RNA polymerase has been reported (11). By analogy, long oligonucleotides hybridized to this strand might also interfere with the elongation phase of transcription. If so, targeted nucleotide exchange by these oligonucleotides could again be enhanced by transcription-coupled repair.

Strand bias in gene repair has also been explained based on the idea that a bias is caused through the orientation of the replication fork as it moves through the coding region of a gene (8). While this explanation cannot be excluded, and there is no reason to assume that it must be mutually exclusive from the transcription scenario, several facts argue against it as the most likely explanation for our results. First, the nontranscribed strand in the plasmid pAURHyg(del)eGFP is the leading strand, while in plasmid pAURHyg(del)eGFP-R, it is the lagging strand. In both cases, the enhancer of repair observed with Hyg3S/74NT is the same. Second, the effect of inducing transcription by growth in the presence of galactose is dramatic: when the gene is significantly down-regulated (repressed), as is the case in the presence of dextrose, and the plasmid pYESHyg(del)eGFP is replicating at a high rate, only minimal strand bias is observed. But when the gene is transcriptionally active, a higher level of gene repair is observed, and the highest level of enhancement is heavily biased toward the nontemplate strand on the same plasmid target, regardless of the status of its replication. These results support previous observations by Leadon and Lawrence (20). The implication that the replication status of the target molecule can account for strand bias was offered recently by Ellis et al. (8). These authors used β protein from bacteriophage λ (12) to promote single-strand DNA annealing of an oligonucleotide (36-mer), presumably to the lagging strand of the target during DNA replication in *E. coli*. We examined this possibility directly, and the data do not support this conclusion. Our results are more consistent with transcriptional activity as the more prominent factor in determining strand selectivity of gene repair. Obvious differences in the systems exist. (i) Ellis et al. (8) carried out their experiments using a very low amount of oligonucleotide vector (200 ng) and do not demonstrate a dosage effect. We examined a wide range of vector amounts but in sharp contrast, at 200 ng, observe no activity. (ii) Ellis et al. (8) use *E. coli* and β protein as a test system, whereas we employ *S. cerevisiae* without the addition of auxiliary factors. However, preliminary data suggest that the addition of eukaryotic recombinases results in higher levels of repair but the strand bias we report is still exhibited. Interestingly, the overexpression of the yeast RAD52 protein, defined as a homolog of β protein by Ellis et al. (8), does not increase targeting frequency in our hands (Liu et al., unpublished data).

We present evidence that RNA polymerase activity can displace the oligonucleotide designed to direct targeted gene repair. Due to the nature of the transcription process and the close proximity in which these events occur, it is unlikely that vectors bound to the template strand are uniquely disrupted; rather, their disruption is more common and more readily detected by our assay systems. As shown in Fig. 5B, evidence to support this notion was obtained in a model episomal system, but recently strand bias for gene repair has been confirmed at the chromosomal level by utilizing a mutant *cyc1* gene in *S. cerevisiae* and a similar single-stranded targeting vector (Brachman and Kmiec, unpublished).

In summary, we have demonstrated a strand bias for targeted gene repair of episomal targets in *S. cerevisiae*. Strand selectivity is measured as a function of the correction of a deletion mutation in a fusion gene. The results of supplemental experiments reveal the same phenomenon when replacement of insertion mutations are corrected by modified single-stranded vectors (data not shown). While several explanations for strand preference can be put forth and are still possible, we present evidence that vector displacement by transcriptional activity is a viable option. Such data are helpful in future design of vectors that can be used in genomics applications where the creation of single-base changes in animal models is the ultimate goal.

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ERRATUM

Strand Bias in Targeted Gene Repair Is Influenced by Transcriptional Activity

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Volume 22, no. 11, p. 3852–3863, 2002. Page 3855: In Fig. 1, "(T)" and "(NT)" and "Hyg3S/74T" and "Hyg3S/74NT" are reversed.

Page 3856: In Fig. 2, the factor "10^5" should not appear in the "Hyg^r" column.

Page 3857: In Fig. 3A, the orientation of the pADH1-HygeGFP-TADH1 cassette is reversed.

Page 3857: In Fig. 3B, the factor "10^5" should not appear in the "Hyg^r" column.