Chemistry & Biology, Vol. 9, 1073–1084, October, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S1074-5521(02)00236-3

Targeted Nucleotide Exchange in *Saccharomyces cerevisiae* Directed by Short Oligonucleotides Containing Locked Nucleic Acids

Hetal Parekh-Olmedo, Miya Drury, and Eric B. Kmiec[1]
Department of Biological Sciences
Delaware Biotechnology Institute
University of Delaware
Newark, Delaware 19716

Summary

Locked nucleic acids (LNAs) are novel base modifications containing a methylene bridge uniting the 2′-oxygen and the 4′-carbon. In this study, LNA-modified single-stranded molecules directed the repair of single base mutations in a yeast chromosomal gene. Using a genetic assay involving a mutant hygromycin-resistance gene, correction of point and frameshift mutations was facilitated by vectors containing an LNA residue on each terminus. Increasing the number of LNA bases on each terminus reduced the correction frequency progressively. When the LNA vector is used in combination with a phosphorothioate-modified vector (74-mer), however, a high level of gene-repair activity occurs; hence, short LNA-based vectors can augment the activity of other types of targeting vectors. These data suggest that oligonucleotides containing locked nucleic acid residues can be used to direct single nucleotide exchange reactions in vivo.

Introduction

Oligonucleotides have been shown to direct the exchange of single nucleotides in genomes from bacteria to human. One of the most promising oligonucleotide vectors is the RNA/DNA chimera, which has been shown to reverse genotype and phenotype in plant and mammalian cells by directing nucleotide replacement or insertion [1–6]. These vectors have also catalyzed the repair of mutations in several animal models [5, 7–9]. Despite these encouraging results, however, the successful application of the "gene repair" or "targeted nucleotide exchange" technique has been restricted, due primarily to the unpredictable level of purity of the chimeric oligonucleotide. Although simple in design, the synthesis of quantitative amounts and the propensity of breakdown products to accumulate have limited its reproducibility and usefulness as a targeting vector.

By its structural nature, the chimera has two fundamental components, a strand of RNA and a strand of DNA, each of which contributes an important function to the gene-repair process [10]. The RNA strand provides an enhanced level of stability once bound to the target site, while the DNA strand directs the majority of the nucleotide exchange [11, 12]. Recently, modified single-stranded, all-*DNA* molecules were shown to be as active as the chimera in mammalian cells [13] and yeast [2, 14] (E. Brachman and E.B.K., submitted). The simplicity of its synthesis and its high cellular transformation efficiency has allowed the single-stranded vector to overcome the barrier of reduced complex stability due to the absence of RNA residues in the vector itself.

Currently, the most active single-stranded vectors are comprised of DNA molecules 25–74 bases in length containing three phosphorothioate linkages at each terminus [11]. The modified linkages protect the vector against nuclease digestion. The advantages of synthetic vectors containing phosphorothioate linkages, however, appear not to translate into mammalian cells or when such vectors are used in clinical trials. First, many reports of oligonucleotide applications (particularly those bearing phosphorothioate modifications) have been generally challenged, and it is now estimated that up to 90% of them appear to contain at least partially unreliable data [15, 16]. This finding may be due to the fact that phosphorothioate linkages have an increased affinity for binding to cellular proteins, resulting in major nonreproducible and nonspecific effects. Second, phosphorothioates produce an elevated level of cellular toxicity [17, 18], reducing their potential applications for functional genomics and/or gene therapy. Thus, the optimal vector design should encompass the following characteristics: an increased level of target affinity, a simple synthesis pathway that produces few breakdown products, and a low level of cellular toxicity.

As mentioned above, we have been developing new generations of single-stranded DNA vectors for targeted nucleotide exchange. In this reaction, the oligonucleotide is introduced into the cell, localizes to its complementary sequence in the genome, and then directs nucleotide exchange at a point or frameshift mutation [19]. Our mechanistic studies [4, 11] have led us to conclude that the two most important factors for successful gene repair are the affinity of the vector for the target site and the stability and nontoxicity of the vector. To meet these requirements, we have begun designing targeting vectors containing locked nucleic acids (LNAs). LNAs are DNA bases containing a methylene bridge between the 2′-oxygen and 4′-carbon, which results in a locked 3′-endo conformation [15] (see Figure 1). These modified bases can be synthesized using standard reagents and are as soluble as RNA or DNA, making them amenable to cell transformation protocols. LNAs also have a high affinity for their complementary sequence [20, 21], although this enhancement is somewhat reduced when paired as short oligos (<10-mer) in the presence of a mismatch. Importantly, however, LNAs show no detectable cellular toxicity, and they are inherently resistant to cellular nucleases [22].

Currently, the most reliable system for testing gene-repair activity is one in which a mutation in the hygromycin-resistant gene in yeast is corrected by modified single-stranded oligomers [2]. The corrected chromosomal gene confers hygromycin resistance onto the cell, a reaction that leads to a phenotypic change, which can subsequently be verified at the DNA level. The goal of

[1]Correspondence: ekmiec@udel.edu

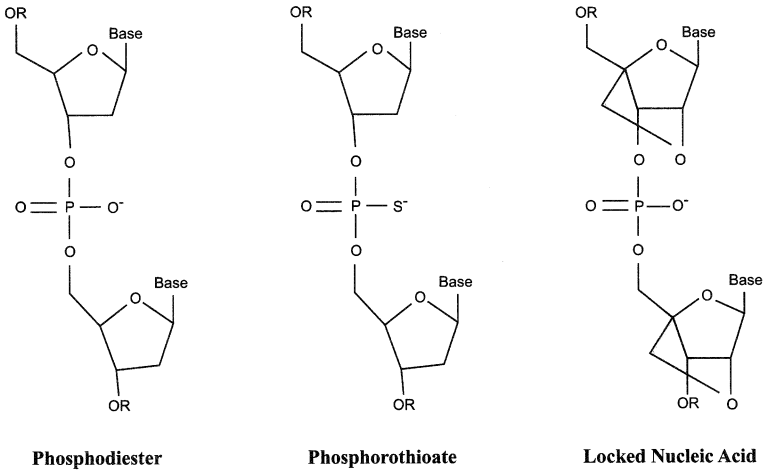


Figure 1. Backbone and Base Modifications of DNA Oligonucleotides Used in This Study

Oligonucleotides designed for gene repair are presented depicting the differences among the modifications.

this study was to test the activity of LNA-containing vectors in this model system by using a molecule that is shorter and thus more reliable in terms of obtaining full-length product at a higher level; perhaps a new vector could be evolved. If successful, LNA-modified vectors could become a useful tool for the modulation of gene expression in animal models. Furthermore, this would be an important step forward for the fields of functional genomics and gene therapy without the concerns of cellular toxicity or aberrant protein binding.

Results

Targeted Nucleotide Exchange Assay System

Saccharomyces cerevisiae is an excellent in vivo model for analyzing structure/function relationships regulating the gene correction reaction. Previous studies have focused on establishing the genetic readout of this system and the strand bias of gene repair [2]. In both of these cases, the target was a mutated hygromycin gene fused to an eGFP gene. A point mutation in the hygromycin gene disables the fusion construct, and only upon nucleotide replacement (G→C), at position 812, will the plasmid confer hygromycin resistance onto the cell. As shown in Figure 2A, the mutation in the fusion gene creates a stop codon, causing the gene to be dysfunctional. This target gene was integrated (two to three copies per genome) into the genome of yeast strain *LSY678*, along with a functional gene conferring resistance to aureobasidin.

Experimentally, the cells are grown to a cell density of approximately 1×10^7 cells/ml, at which point the oligonucleotide vectors are introduced into the cells by electroporation. No significant differences in the transformation efficiencies of any vectors used in this study were observed, as evaluated using ^{32}P-labeled modified oligonucleotides (data not shown, but see [14]). Cells were allowed to recover for 3 hr in media without selection and then spread on agar plates containing either hygromycin or aureobasidin, the latter of which serves as an internal control to normalize for cell viability after electroporation. Colony counts are obtained for each selection, and the correction efficiency is determined by dividing the number of colonies that are hygromycin resistant by those that are aureobasidin resistant per 10^5 cells plated.

Vectors Containing Locked Nucleic Acid Direct Targeted Nucleotide Exchange

In earlier studies aimed at evaluating the biochemical nature of targeted repair, Gamper et al. [11] found that the rate-limiting step in the reaction was the half-life of the joint molecule formed between the oligonucleotide vector and the target site within the helix. Specifically, the half-life of the intermediate was extended when vectors containing a chimeric composition of RNA and DNA residues were used. However, while these chimeric structures enabled a longer half-life, the RNA residues appeared to interfere with the correction process and thus decrease correction frequency. In addition, chimeric vectors are difficult to synthesize as full-length molecules, and breakdown (N-1, N-2…) products abound. Based on these data, our goal was to identify a short oligonucleotide vector that would bind in a more stable fashion and at the same time direct comparable levels of gene repair. But it also needed to be nuclease resistant and exhibit a lower level of cellular toxicity than phosphorothioate-modified oligonucleotides. Thus, the locked nucleic acid residue modification was appealing since it possesses many of these characteristics and can be incorporated easily into short molecular vectors.

To test the activity of an LNA-modified vector in the nucleotide-replacement reaction, Hyg10, consisting of 24 bases with the terminal residues being LNA, was designed and synthesized (Figure 2A). Vector Hyg10NT contains one LNA base modification on each terminus and is complementary to the nontranscribed strand, while Hyg10T is identical in structure but is complementary to the transcribed strand. In both cases, a mismatched base pair is created at the site of the targeted mutation. Two control molecules were used: Hyg10NT*, which is identical to Hyg10NT, except it forms a perfect match with the nontranscribed strand, and Hyg10T*, which is completely complementary to the transcribed strand. These vectors were electroporated into *LSY678-(Int)Hyg*, and the level of gene repair was measured as a function of hygromycin and aureobasidin resistance. As shown in Figure 2B, the LNA-modified vectors sup-

A. **Target and vectors for nucleotide exchange of repair of a hygromycin point mutation.**

Wild type

```
5´... GATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGC...3´
3´... CTACATCCTCCCGCACCTATACAGGACGCCCATTTATCGACG...5´
       D  V  G  G  R  G  Y  V  L  R  V  N  S  C
```

Point Mutation

```
5´... GATGTAGGAGGGCGTGGATAGGTCCTGCGGGTAAATAGCTGC...3´
3´... CTACATCCTCCCGCACCTATCCAGGACGCCCATTTATCGACG...5´
       D  V  G  G  R  G  *
```

Hyg10NT
5´ +AdCdCdCdGdCdAdGdGdAdCdGdTdAdTdCdCdAdCdGdCdCdC+T 3´
24-mer, 1 LNA residue on each end; targets non-transcribed strand.

Hyg10T
5´ +AdGdGdGdCdGdTdGdGdAdTdAdCdGdTdCdCdTdGdCdGdGdG+T 3´
24-mer, 1 LNA residue on each end; targets transcribed strand.

Hyg10NT*
5´ +AdCdCdCdGdCdAdGdGdAdCdCdTdAdTdCdCdAdCdGdCdCdC+T 3´
24-mer, 1 LNA residue on each end, perfectly matched to the nontranscribed strand.

Hyg10T*
5´ +AdGdGdGdCdGdTdGdGdAdTdAdGdGdTdCdCdTdGdCdGdGdG+T 3´
24-mer, 1 LNA residue on each end, perfectly matched to the transcribed strand.

Kan70T
5' C*A*T*CAG AGC AGC CAA TTG TCT GTT GTG CCC AGT CGT AGC CGA ATA GCC TCT CCA CCC AAA CGG CCG G*A*G*A 3'
70-mer, non-specific control.

B. **Nucleotide repair in *LSY678(Int)Hyg.***

Vector	*Amount(μg)*	*Hyg^r*	*Aureo^r*	*C.E.(x10^{-5})*
Hyg10T	1.6	0	250	0.0
Hyg10T	6.4	4	241	0.016
Hyg10T	12.9	8	270	0.033
-	0	0	299	0.0
Hyg10NT	1.6	129	272	0.47
Hyg10NT	6.4	796	213	3.74
Hyg10NT	12.9	648	193	3.38
Hyg10NT*	12.9	0	243	0.0
Hyg10T*	12.9	0	289	0.0
Kan70T	12.9	0	270	0.0

C. **DNA sequence analyses of converted clones.**

Figure 2. The Targeting Assay System and Correction by LNA-Modified Oligonucleotides

(A) *LSY678(Int)*pAurHyg(rep)eGFP contains a mutation at nucleotide 137 (TAT→TAG) eliminating the hygromycin-resistance phenotype. DNA sequences of single-stranded oligonucleotides with one LNA residue on each end that target the transcribed (T) and nontranscribed (NT) strands are depicted. Oligonucleotides that are perfectly matched (indicated by asterisks) to the transcribed and nontranscribed strands were designed as controls; they form no mismatch with the target. Kan70T, a 70-mer containing three phosphorothioate linkages on both termini, is used as a nonspecific control.

(B) Various concentrations of targeting oligonucleotides were electroporated into *LSY678 (Int)*pAurHyg(rep)eGFP and grown for 3 days at 30°C. Correction efficiency was calculated by dividing the number of hygromycin-resistant colonies by the number of aureobasidin-resistant colonies (per 10^5). These results represent an average of four independent experiments with a variance of ±10%. Hyg^r, number of hygromycin-resistant colonies; Aureo^r, number of aureobasidin-resistant colonies.

(C) Single colonies were picked from hygromycin plates and amplified by colony PCR. Conversion of the hygromycin mutation was confirmed by sequencing the PCR products using an automated ABI 310 capillary sequencer. Wild-Type Sequence, the sequence of the DNA prior to mutagenesis; Mutated Sequence, sequence of the mutated target without treatment; Converted Sequence, sequence of converted clone amplified by colony PCR from a hygromycin plate.

port gene-repair activity at nearly each dosage. The Hyg10 molecules also exhibit the characteristic strand-bias effect reported earlier [2]: vectors designed to hybridize to the nontranscribed strand produce a higher level of gene repair than those complementary to the transcribed strand. For example, at 6.4 μg, the bias for gene repair is 300× in favor of Hyg10NT. In addition, the level of repair induced by Hyg10NT compares favorably with the level directed by the phosphorothioate-modified vector, Hyg3S/74NT [2], albeit at a different dosage with respect to molar amounts. The oligomers that are designed to form a perfect match with either strand of the target site (Hyg10T* and Hyg10NT*) directed no gene-repair activity. Independent confirmation of the correction of the hygromycin mutation came from direct DNA sequence analyses of DNA isolated from hyg^r colo-

A. Sequence of LNA-modified vectors.

Hyg10NT/3'
5′ AdCdCdCdGdCdAdGdGdAdCd**G**dTdAdTdCdCdAdCdGdCdCdC+T 3′
24-mer, 1 LNA residue on the 3′ end.

Hyg11
5′ +A+CdCdCdGdCdAdGdGdAdCd**G**dTdAdTdCdCdAdCdGdCdC+C+T 3′
24-mer, 2 LNA residues on each end.

Hyg12
5′ +A+C+CdCdGdCdAdGdGdAdCd**G**dTdAdTdCdCdAdCdGdC+C+C+T 3′
24-mer, 3 LNA residues on each end.

Hyg13
5'-+G+G+C +G+CdA dGdCdT dAdTdT dTdAdC dCdCdG dCdAdG dGdAdC d**G**dTdA dTdCdC dAdCdG dCdCdC dTdCdC dTdAdC dAdTdC +G+A+A +G+C -3'
50-mer, 5 LNA residues on each end.

Hyg14
5'-+G+T+A+G+AdA dAdCdC dAdTdC dGdGdC dGdCdA dGdCdT dAdTdT dTdAdC dCdCdG dCdAdG dGdAdC d**G**dTdA dTdCdC dAdCdG dCdCdC dTdCdC dTdAdC dAdTdC dGdAdA dGdCdT dGdAdA dAdGdC +A+C+G +A+G -3'
74-mer, 5 LNA residues on each end.

B. Increasing the number of labeled nucleic acid residues in an oligonucleotide vector reduces repair activity.

Vector	*Amount(μg)*	*Hyg^r*	*Aureo^r*	*C.E.(x10^{-5})*
Hyg10NT/3′	0	0	0	0.0
Hyg10NT/3′	1.6	112	331	0.34
Hyg10NT/3′	6.4	421	404	1.04
Hyg10NT/3′	12.9	534	246	2.2
Hyg11	1.6	22	261	0.08
Hyg11	6.4	30	260	0.11
Hyg11	12.9	81	296	0.27
Hyg12	1.6	6	460	0.013
Hyg12	6.4	38	355	0.11
Hyg12	12.9	36	291	0.12
Hyg13	12.9	92	255	0.36
Hyg14	20	155	158	0.98

Figure 3. Increasing the Number of LNA Residues on the Vector Reduces Repair Efficiency

(A) 24-mer oligonucleotides with one LNA residue on the 3′ end (Hyg10NT/3′), two LNA residues (Hyg11) on each end, or three LNA residues (Hyg12) on each end, all targeting the nontranscribed strand of hygromycin gene, were designed. DNA bases are indicated by "d" in the single-stranded vectors, where the mismatch is centered, keeping the length of the oligonucleotide constant, and "+" indicates an LNA residue.

(B) A dose response is depicted for each type of vector used in the experiment. Correction efficiency (C.E.) is determined for the indicated amounts of each vector to the yeast. Gene repair activity (per 10^5) is calculated as a correction efficiency (C.E. $\times$ 10^{-5}) with a variance of $\pm$15% (average of four experiments). Hyg^r, number of hygromycin-resistant colonies; Aureo^r, number of aureobasidin-resistant colonies.

nies. The results (Figure 2C) indicate that the base targeted for alteration was changed specifically.

To extend this observation, we designed DNA vectors containing various numbers of LNA-modified bases and tested them in the same repair assay. The first structural alterations included changes in the number of LNA bases contained within each molecule (Figure 3A). Vector Hyg10NT/3′ is identical to Hyg10NT, except it contains a single LNA at its 3′ end, as opposed to the original, which had an LNA residue on each end. Hyg11NT and Hyg12NT were identical in sequence to Hyg10, but contained either two (Hyg11NT) or three (Hyg12NT) LNA bases on each end. All three new constructs were designed to be complementary to the nontranscribed strand of the target. When tested for targeted nucleotide exchange in the integrated hygromycin system, each vector was found to be competent for the reaction (Figure 3B). Hyg10NT/3′ displayed slightly lower levels of repair activity to that observed with Hyg10NT. In contrast, Hyg11 and Hyg12 produced substantially lower levels of gene repair. Thus, a general trend toward reduced activity is apparent as the number of LNA bases at each terminus increases from one to two and finally to three. Since the length of the vector remained the same, the only structural change is the ratio of LNA bases to natural DNA bases. Thus, an increase in LNA modifications within a fixed vector length reduces gene repair activity, similar to the in vitro data reported by Gamper et al. [11] using phosphorothioate-modified or 2′-O methyl RNA-containing oligonucleotides.

We examined this finding further by designing vectors with a greater number of LNA residues on each end. The first, Hyg13, was 50 bases in length and contained five LNA residues, while the second, Hyg14, was a 74-mer, again with five LNA residues on each end; both target the nontranscribed strand (Figure 3A). These numbers were chosen because the percentage of LNA residues in the overall molecule is in the same range (16%–20%) as Hyg11. When Hyg13 and Hyg14 were tested for gene repair activity, both were found to be able to direct correction (Figure 3B). While the level of correction was lower than that produced by Hyg10NT, each did exhibit a higher level of repair than Hyg11; Hyg13 was three times as effective, while Hyg14 was nine times as effective. In essence, it appears that the

A. Target sequence of the Hyg(Δ) allele and oligonucleotides Hyg10NT and Hyg3S/25NT.

```
      Wild type

5´... GATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGC...3´
3´... CTACATCCTCCCGCACCTATACAGGACGCCCATTTATCGACG...5´
       D  V  G  G  R  G  Y  V  L  R  V  N  S  C

      Deletion

5´... GATGTAGGAGGGCGTGGATAGTCCTGCGGGTAAATAGCTGC...3´
3´... CTACATCCTCCCGCACCTATCAGGACGCCCATTTATCGACG...5´
       D  V  G  G  R  G  *
```

Hyg10NT
5´+AdCdCdCdGdCdAdGdGdAdCdGdTdAdTdCdCdAdCdGdCdCdC+T 3´

Hyg3S/25NT
5´T*A*C*CCGCAGGACGTATCCACGC*C*C*T 3´

B. Nucleotide repair of a deletion mutant.

Vector	*Amount(μg)*	*Hyg^r*	*Aureo^r*	*C.E.(x10^{-5})*
Hyg10NT	1.6	104	278	0.37
Hyg10NT	6.4	341	330	1.03
Hyg10NT	12.9	313	256	1.22
Hyg3S/25NT	1.6	49	280	0.175
Hyg3S/25NT	6.4	144	344	0.42
Hyg3S/25NT	12.9	137	223	0.61

Figure 4. Gene Repair of a Frameshift Mutation

(A) The sequence of the deletion mutation at nucleotide 137 (indicated by asterisks) in *LSY678(Int)*pAurHyg(Δ)eGFP is displayed. This mutation (TAΔ→TAC) is targeted for correction by a 24-mer LNA-modified single-stranded oligonucleotide (Hyg10NT) or a 25-mer vector (Hyg3S/25NT) containing three phosphorothioate linkages on each end.

(B) The vectors were electroporated into the deletion strain and plated on hygromycin and aureobasidin plates. Colony numbers and correction efficiency (C.E. $\times 10^{-5}$) were determined as an average of four experiments with a variance of ±15%). Hyg^r, hygromycin-resistant colonies; Aureo^r, aureobasidin-resistant colonies.

level of nucleotide exchange is influenced by either the length of the region containing phosphodiester bonds in the targeting vector or the number of modified residues in the whole molecule. These results are again consistent with our previous data (Figure 3) [12].

To test the repair activity of these vectors on a frameshift mutation, a single base deletion in the hygromycin gene at the same site as the replacement [2] was used as the target for gene repair. This integrated mutant gene (Figure 4A) served as a target for repair by Hyg10NT, the same vector used for point mutation repair, and a 74-mer with two LNA bases on each end, for correction of the deletion mutant. As shown in Figure 4B, both Hyg10NT and Hyg3S/25NT can direct gene repair at each dosage with an approximately 2-fold difference in activity between the vectors. As reported previously [2], correction of a frameshift mutation is significantly lower than the efficiency of repair of a point mutation. DNA sequence analyses confirm that the proper base was inserted at the precise location (data not shown).

Oligonucleotide vectors have been designed such that either strand of the helix can be targeted. In all cases, regardless of the molecular composition of the vector, a strand bias was observed with higher frequencies of gene repair being observed with oligos complementary to the nontranscribed strand. But no data exist for a dual targeting approach; that is, an experiment wherein vectors designed to target either the transcribed or nontranscribed strand are used together. Hence, we explored the potential of increasing gene frequencies by using such a dual targeting approach. Previous results indicated that 74-mers directed against either strand of the hygromycin mutant sequence were the most active in directing repair [2]. First, we tested these vectors individually for gene repair activity to establish a baseline of correction. As shown in Table 1, strand bias of correction is observed with the Hyg3S/74NT and Hyg3S/74T vectors, favoring Hyg3S/74NT, confirming the results of Liu et al. [2]. Comparatively, the Hyg3S/74NT vector is slightly more efficient than Hyg10NT at this particular dosage (5 μg Hyg3S/74NT is equimolar to 1.6 μg Hyg10NT), also as shown in Table 1. When equal molar amounts of these two vectors were combined in a dual repair experiment, Hyg3S/74NT and Hyg10T did not elevate the correction efficiency above the level directed by Hyg3S/74NT alone. Combining the Hyg3S/74NT or Hyg3S/74T vectors with a Hyg10 molecule (Hyg10T* or Hyg10NT*) that anneals with perfect complementarity did not elevate the level of gene repair. The pair of Hyg3S/74T and Hyg10NT, however, produced a significant increase in correction efficiency when used as dual targeting vectors. Replacing Hyg10NT with Hyg11 or Hyg12 in combination with Hyg3S/74T eliminated the enhancement entirely, suggesting a requirement for a low number of LNA residues

Table 1. Nucleotide Repair Using Multiple Single-Stranded Oligonucleotides

Vector(s)	Amount (μg)	Correction Efficiency ($\times 10^{-5}$)
A. Hyg3S/74T	5.0	0.22 ± 0.3
B. Hyg3S/74NT	5.0	0.89 ± 1.3
C. Hyg10T	1.6	0.03 ± 0.0005
D. Hyg10NT	1.6	0.65 ± 0.09
E. Hyg10T*	1.6	0 -
F. Hyg10NT*	1.6	0 -
G. Hyg3S/74T	5.0	
Hyg10NT	1.6	3.98 ± 0.59
H. Hyg3S/74T	5.0	
Hyg10NT*	1.6	0.15 ± 0.02
I. Hyg3S/74NT	5.0	
Hyg10T	1.6	0.32 ± 0.04
J. Hyg3S/74NT	5.0	
Hyg10T*	1.6	0.49 ± 0.07
K. Hyg3S/74T	5.0	
Hyg3S/25NT	1.6	0.61 ± 0.11
L. Hyg3S/74T	5.0	
Hyg11NT	1.6	0.75 ± 0.10
M. Hyg3/74T	5.0	
Hyg12NT	1.6	0.46 ± 0.06
N. Hyg3S/74T	5.0	
Hyg3S/74NT	5.0	0.34 ± 0.05

Modified DNA vectors designed to direct conversion of the transcribed or nontranscribed strand were electroporated into *LSY678* (*Int*)pAURHyg(rep)eGFP. Hygromycin-resistant colonies and aureobasidin-resistant colonies were quantitated after plating on appropriate media. The frequency of correction (per 10^5) is determined by dividing the hyg^r colony number by the aur^r colony number. These numbers represent an average of four experiments with the standard deviation indicated for correction efficiency.

in the shorter vector. The results obtained with the combination of Hyg3S/74T and Hyg10NT could be viewed in two ways: first, the presence of Hyg10NT increases the potency of Hyg3S/74T over 17-fold, or second, the presence of Hyg3S/74T elevated repair frequency directed by Hyg10NT over 8-fold. Furthermore, replacing Hyg10NT with vectors containing two or three LNA residues on each end reduces the overall correction efficiency proportionally. These results are consistent with the drop in repair activity observed when Hyg10, Hyg11, or Hyg12 is used as the sole vector in the reaction (see Figure 2B). Taken together, these data suggest that a unique pair of dual repair vectors can significantly increase correction efficiency.

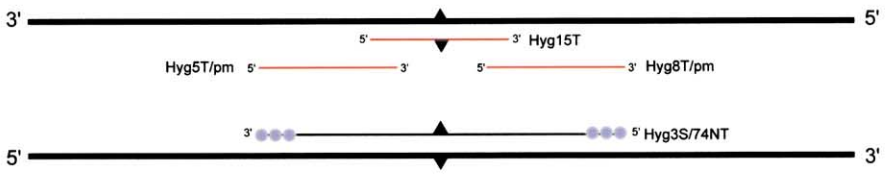
The data presented in Figure 3 suggest that the number of LNA residues in an oligonucleotide can influence the frequency of targeted nucleotide exchange. It has been postulated that the helical pitch of the DNA duplex is altered in regions where DNA bases are paired with LNA residues and that this configuration favors a higher degree of base stacking [23]. Furthermore, this stacking results in a conformation that resembles RNA-DNA hybrids, which have been shown to serve as ineffective templates for targeted nucleotide exchange [11, 12]. Thus, a reduction in nucleotide-exchange frequency is at least predicted when the number of LNA residues is increased. To further test this hypothesis, we created a 15-mer containing 12 LNA residues that could direct nucleotide exchange on the T strand. Nucleotide repair using Hyg15T did not take place, as no hygromycin-resistant colonies were observed (Figure 4). Hyg15T was designed specifically with three DNA residues to insure that the target base and two adjacent bases could be used as a template during the reaction and that "DNA pairing" could actually occur. But even under these conditions, the results suggest that Hyg15T is nonfunctional. We concluded, therefore, that single-stranded oligonucleotide vectors containing greater than 16%–20% LNA residues are biologically inert.

Next, we tested if Hyg15T could exert a negative effect on nucleotide exchange in *trans* by coupling it with Hyg74NT in a dual targeting event. We had shown previously that certain combinations produce a high frequency of repair, but as shown in Figure 5B, a low level of repair was observed when the combination of Hyg74NT/Hyg15T was used, as compared to Hyg74NT alone. Thus, as is the case with Hyg11 and Hyg12, the presence of Hyg15T acts in a negative fashion and reduces the frequency of targeted nucleotide exchange when paired with Hyg3S/74NT.

Finally, we designed two 12-mers containing all LNA residues that would bind with perfect complementarity to a target site. In this case, however, Hyg5T/pm was designed to hybridize to a region 5′ at nucleotide 119 relative to the site of repair, while Hyg8T/pm was designed to bind on the 3′ side of the target base at nucleotide 143. Both vectors can still partially hybridize to Hyg3S/74NT (see Figure 5A). Essentially, these two vectors are positioned off-center relative to the site of nucleotide exchange, which is directed by Hyg3S/74NT, while being perfectly matched to the surrounding DNA sequence (see Figure 5A). They also contain the same number of LNA residues [12] as Hyg15T. As expected, when either of these oligonucleotides is electroporated into LSY678(Int)pAURHyg(rep)eGFP no hygromycin-resistant colonies are formed, and thus the correction efficiency is 0.0. But when either is coupled with Hyg3S/74NT in a dual targeting experiment, a correction efficiency rivaling the one observed with Hyg74NT alone is observed. For example, the combination of Hyg3S/74NT and Hyg5T/pm is 7-fold more active than the combination of Hyg3S/74NT and Hyg15T. Thus, an oligonucleotide comprised of all LNA residues does not inhibit nucleotide exchange if positioned 5′ or 3′ relative to a second oligonucleotide directing the nucleotide exchange reaction.

The source of the different repair frequencies among the combination vector systems depicted in Table 1, G, I, and K, respectively, could be in the initialization phase of gene repair. In this part of the reaction, the DNA vectors pair with the target and hybridize to their cognate complementary sequence. Recently, we proposed that the structure of this reaction intermediate is double-D-loop [24], a configuration in which each partner aligns in homologous register with a recipient strand of the duplex. It is possible that various pairs of targeting vectors assemble into the D-loop structure with different efficiencies. Figure 6A depicts the structures of single- and double-D-loops as reaction intermediates in the gene-repair pathway. And thus we tested (double) D-loop formation using selected sets of DNA vectors and a superhelical plasmid bearing complementarity to each vector. Previously, we had established conditions

A.



Hyg15T

5' +G+TdG+G+A+TdA+**C**+G+TdC+C+T+G+C 3'
15-mer, 12 LNA residues, targets the transcribed strand.

Hyg5T/pm

5' +T+G+T+A+G+G+A+G+G+G+C+G 3'
12-mer, all LNA residues, perfectly matched to the transcribed strand at nucleotides 119-130.

Hyg8T/pm

5' +G+C+G+G+G+T+A+A+A+T+A+G 3'
12-mer, all LNA residues, perfectly matched to the transcribed strand at nucleotides 143-154.

B.

		Colonies		
Vector	Amount (pmols)	Hyg^r	$Aureo^r$	Corr.Eff.($x10^{-5}$)
Hyg15T	220	0	367	0.0
Hyg3S/74NT	220	552	251	1.33
Hyg5T/pm	220	0	273	0.0
Hyg8T/pm	220	0	299	0.0
Hyg3S/74NT Hyg15T	220 220	90	339	0.17
Hyg3S/74NT Hyg5T/pm	220 220	555	466	1.19
Hyg3S/74NT Hyg8T/pm	220 220	278	275	1.01

Figure 5. Location of LNA-Modified Oligonucleotide Vectors in Dual Targeted Reaction Influences the Frequency of Nucleotide Repair

(A) The positions of the oligonucleotide vectors containing LNA residues are diagrammed in red, relative to the template strand of the mutated hygromycin gene. Hyg3S/74T is a 74-mer with three phosphorothioate linkages on each termini; Hyg15T (15-mer) is complementary to the template strand and can form a single mismatch; Hyg5T/pm (12-mer) is totally complementary to the template strand 5′ relative to the position of Hyg3S/74NT; Hyg8T/pm (12-mer) is totally complementary to the template strand near the 3′ end of the Hyg3S/74NT. The triangle indicates the position of the mismatch, and the blue circles represent phosphorothioate linkages.

(B) The number of hygromycin-resistant colonies was measured after the indicated vectors were electroporated into LSY678(Int)pAURHyg(rep)-eGFP. Results were tabulated in the same fashion as described for Table 1.

for D-loop formation and had carried out a series of studies aimed at characterizing its formation and stability [12]. Plasmid pAURHyg(rep)eGFP was used as the target, the same construct used for genomic correction, and the RecA protein, known to promote efficient D-loop formation, was employed to catalyze the reaction. D-loop formation was measured by the assimilation of ^{32}P-labeled, single-stranded DNA molecules into the superhelical plasmid followed by agarose gel electrophoresis [25]. The product band in the gel was quantified using ImageQuant 5.2 software after visualization on a phosphoimager. As shown in Figure 6B, D-loop formation is dependent on all components of the reaction being present (vectors, plasmids, and RecA protein). In

A. Single and Double D-loops

B. D-loop Formation with Various Oligonucleotides

Vector(s)	Reaction Conditions	% Joint Formation
Hyg3S/74T + Hyg10NT/3'	No plasmid	0.0
Hyg3S/74T + Hyg10NT/3'	No RecA	0.0
Hyg3S/74NT + Hyg10T	Complete	0.99
Hyg3S/74T + Hyg10NT/3'	Complete	0.90
Hyg3S/74T + Hyg3S/25NT	Complete	1.64
Hyg3S/74NT	No RecA	0.0
Hyg10T	Complete	0.0
Hyg10NT/3'	Complete	0.0
Hyg3S/25NT	Complete	0.0
Hyg3S/74NT	Complete	9.28
Hyg3S/74T	Complete	2.72

Figure 6. D-Loop Formation Occurs with All Dual-Repair Vectors

(A) The structure of single- and double-D-loops is provided in schematic form to illustrate the interwinding of one or two oligonucleotide vectors with the target site.

(B) Double-D-loop joint molecule formation. Presynapsis of joint molecules were formed by incubating the incoming oligonucleotide of 74 base pairs with RecA. This complex was then incubated with plasmid pAURHyg(rep)-eGFP followed by incubation with an annealing oligonucleotide 25 base pairs in length. In this case, the annealing oligonucleotide was labeled using ^{32}P to detect formation of the double-D-loop joint. Presynapsis of joint molecules were formed by incubating the incoming oligonucleotide with RecA. This complex was then incubated with plasmid pAURHyg(rep)eGFP to form a D-loop joint. The incoming oligonucleotide was radiolabeled with ^{32}P to detect joint formation. Quantification of D-loop and double-D-loop complex formation was carried out using ImageQuant 5.2 software after visualization using a Typhoon 860 Phosphoimager (Molecular Dynamics Inc., CA).

the reaction, the 74-mer, known as the incoming oligo, is left unlabeled (as is the plasmid), while the 24-mer or 25-mer, known as the annealing oligo, is tagged with a ^{32}P label. This insures that the assimilation of the annealing oligo, a sign of double-D-loop formation, is traced. And in each case a reaction product is observed, indicating that similar levels of double-D-loop form with each set of vectors. Annealing vectors (24-mers or 25-mers) do not form D-loops (single) in the absence of the incoming 74-mer, while the 74-mer alone assimilates efficiently into the superhelical plasmid. These results suggest that double-D-loop formation can occur with various combinations of vectors independent of their capacity to catalyze gene repair.

Discussion

The central goal of this study was to explore the possibility that synthetic oligonucleotides containing locked nucleic acids can direct gene repair in *Saccharomyces cerevisiae*. LNA-modified vectors have a number of characteristics that make them appealing for future applications in functional genomics and gene therapy. Among these are their low level of cellular toxicity and their elevated levels of intracellular stability. We were also interested in identifying a shorter vector that enabled the same level of gene repair activity as the Hyg3S/74NT series [2].

The target in this study was a mutated hygromycin gene integrated into strain *LSY678* [2, 14]. The vectors were designed to target either strand of the gene while hybridizing to the site with the mismatched base pair located in the center of the D-loop structure. The nucleotide exchange event can then be catalyzed by proteins involved in both DNA recombination and DNA repair pathways. Our results demonstrate, in fact, that a 24-mer oligonucleotide with a single LNA residue at either one end or both ends is capable of directing the repair of point and frameshift mutations. The level of correction approximates that observed for the phosphorothioate-modified 74-mer, and as reported previously [2, 13, 14], the correction of a frameshift mutation occurs at a lower frequency than the repair of a point mutation. It is important to note that the target gene in this case has been inserted via homologous recombination into the chromosomal site with Southern blot analyses indicating only one or two copies of this mutated gene being present (data not shown). Thus, in terms of copy number, the target reflects the normal environment of a chromosomal gene. It is possible, however, that a "native gene"

under the control of its own endogenous promoter could be less or more amenable to the repair event.

Strand bias for gene repair using the LNA-modified 24-mer (Hyg10) was also observed, favoring those vectors that are complementary to the nontranscribed strand. The preference for the nontranscribed strand is likely based, at least in part, on the transcriptional activity of the target gene. While others have suggested that replication may play a crucial role in determining which strand is more efficiently corrected [26], recent data from Liu et al. [25] demonstrate the importance of transcription. In this case, the oligonucleotide bound to the transcribed strand is displaced by the movement of the RNA polymerase and the production of mRNA. In contrast, the vector designed to hybridize to the nontranscribed strand is not displaced by the movement of the polymerase. The increased level of binding affinity conferred on Hyg10NT by the LNA residue(s) may also help stabilize this pairing and help explain the hundredfold difference in strand bias between Hyg10T and Hyg10NT (see Figure 1B). DNA sequence analyses of corrected colonies reveal that precise nucleotide exchange occurred in all reactions directed by Hyg10NT.

The mispair in the Hyg10NT-helix joint complex site is a G/G transversion, while the Hyg10T-driven complex is C/C transversion, both of which are repaired with moderate efficiency in yeast [27]. Thus, it is unlikely that the specific mismatch created by either Hyg10T or Hyg10NT contributes to the bias of repair. Recent results from experiments using similar oligonucleotides also confirm that transversions are repaired with similar efficiency in *Saccharomyces cerevisiae* (E. Brachman and E.B.K., submitted). We cannot, however, rule out the possibility that nicks in the chromosome near the mismatched base influence the preference of base repair, although such an effect would most likely impact plasmid-borne mismatches more directly [27].

The frequency of gene repair directed by these vectors is sensitive to the number of LNA residues on each end. When Hyg10NT/3′, an oligonucleotide with a single LNA at the 3′ end of the vector, is used, the level of repair is approximately two-thirds or greater the activity of Hyg10NT. This is not the case, however, when the number of terminal LNAs increases from one to two to three, respectively. Several explanations are possible for these observations, including the fact that Hyg11 or Hyg12, containing two or three LNAs on each end, may bind with an increased affinity to the DNA target [22]. Such a scenario would imply that Hyg11 or Hyg12 does not dissociate but somehow impairs the repair reaction by its presence. Alternatively, the reduction in activity exhibited by Hyg11 or Hyg12 may be due to the increase in the number of LNA residues on each end. A similar phenomenon was observed when the number of phosphorothioates in the 25-mer was increased from three to six to nine, respectively [11]. It is plausible that a certain length of unmodified bases needs to exist to enable vector-directed DNA repair, and perhaps LNA residues encroach on this "region of repair." Elegant work by Nielson et al. [28] established that LNA molecules hybridized to DNA exhibit a high stability due in all likelihood to a local change in the geometry of the phosphate backbone. This higher degree of stacking creates a duplex molecule bearing structural resemblance to one that contains RNA. We have shown previously that increasing the number of RNA residues in a single-stranded targeting vector reduces the frequency of repair [11]. The data presented herein using LNA residues support that observation. While the mechanism of targeted gene repair directed by oligonucleotide vectors has only been partially elucidated [10–12, 29], it is clear that members of the mismatch repair pathway play significant roles. As such, the binding of the Msh2 complex to the heteroduplex site initiates downstream events that lead to repair [30]. Since binding at distal sites initializes the repair process, the presence of the LNA residues may deter appropriate protein-DNA interactions by altering the structure of any of the specific protein-DNA interactions. This, in turn, would abort the repair event because the "spacing" between the Msh2 complex and the mismatch is not optimal. Because the reduction in gene repair also appears to be correlated with the number of LNA residues at each terminus, it is plausible that initialization of the repair event is impaired by altered DNA structure.

Nucleotide exchange directed by two single-stranded vectors improves the frequency of repair over the activity exhibited by a single vector, but only when Hyg10, Hyg11, or Hyg12 are paired with a 74-mer that targets the transcribed strand (Hyg3S/74T). Previous data had shown the Hyg3S/74T has a lower capacity to direct gene repair than its counterpart Hyg3S/74NT [2, 25]. But specifically within this group, only one pair, Hyg3S/74T and Hyg10NT, produce a substantial increase: 17-fold above the activity of Hyg3S/74T. The specificity of dual targeting partners is surprising, and no clear explanation has arisen from the various experiments outlined herein. The possibility that the same vector combinations are not assimilated into the double-D-loop reaction intermediate has been weakened by biochemical data (see Figure 6). It is possible, however, that the two vectors anneal prior to entering the nucleus and that the stability of the partial duplexes vary based on the composition of the shorter partner. Duplex molecules containing at least one LNA-modified oligonucleotide have a higher level of stability than a similarly sized oligonucleotide containing the phosphorothioate modification [31]. Such stability would enhance the reaction at either the partial-duplex stage (providing a more intact substrate for the recombinase) or within the double-D-loop itself (lengthening its half-life).

When one of the pair of oligonucleotides contains high numbers of LNA residues and is located in the center, the level of nucleotide exchange is reduced substantially. Yet positioning the LNA vector 5′ or 3′ relative to the targeting oligonucleotide does not inhibit the repair reaction. These observations suggest that the stability of duplex targeting vehicles may be one critical feature in determining the frequency of nucleotide exchange. In a pure system, each LNA residue can raise the temperature of a single duplex approximately 5°C [32, 33]. Thus, as the number of LNA residues increases from 2 (Hyg10) to 4 (Hyg11) to 6 (Hyg12) to 12 (Hyg15T, Hyg5T/pm, and Hyg8T/pm), the stability of the duplex structure is increased significantly. We cannot simply assume that such rules apply in vivo, but it is likely that any duplex

structure formed in this reaction will dissociate more slowly. From an experimental standpoint, there are two obvious places where a stable duplex may interfere with the gene-repair process. First, the two oligonucleotides are coelectroporated into the cell, and thus an annealing reaction between the vectors could take place prior to entering the target site. Second, the assimilation of two vectors into the target site may afford the opportunity for the vectors to pair to one another rather than the target strands. In this case, annealing will essentially promote a reversal reaction and eliminate the paired vector duplex from the reaction intermediate. Hence, any modification, such as an LNA residue, which promotes a more stable duplex vector pair will reduce the propensity for DNA repair to occur by simply eliminating the repair complex from binding properly. As such, Hyg11, Hyg12, and Hyg15T have been observed to reduce proportionally the level of nucleotide repair. There are, however, two caveats to this explanation. By positioning the LNA vector at the 3′ or 5′ side relative to its partner vector, the repair reaction is restored. In addition, the use of a single LNA residue on each terminus of the LNA vector coupled with a phosphorothioate-modified oligonucleotide perhaps provides enough stability to stabilize any potential duplex, but permits dissociation as the reaction intermediate is formed and the repair phase of the reaction ensues. Alternatively, the number of LNA residues may simply affect the repair phase directly by changing the stacking of any DNA strand to which it is bound, as discussed above.

Recently, Mazin et al. [34] demonstrated that duplex molecules with such terminal overhangs are excellent substrates for DNA pairing events catalyzed by a DNA recombinase. Thus, by their very nature combinations containing LNA-modified vectors would create a larger population of these partially duplexed substrates. In addition, the binding of Hyg10T to the transcribed strand occurs with higher affinity than that of Hyg3S/74T due to the presence of the LNA residues [31]. This binding could lower the level of transcription by blocking the movement of the RNA polymerase and as a consequence reduce the capacity of the cell to exhibit hygromycin resistance. Alternatively, strands that are bound to the transcribed strand of the target are displaced by polymerase movement [25]. As mentioned above, however, the increased affinity of LNA oligonucleotides for complementary, unmodified DNA strands is reduced in the presence of a mismatched base, so this possibility needs more testing.

In summary, we have demonstrated the utility of LNA-modified, single-stranded DNA vectors in targeted gene repair. The nontoxic, nuclease-resistant characteristics of this base modification are attractive for many molecular applications. The versatility of these vectors in directing the correction of both point and frameshift mutations may now be exploited in other systems where the aim is to reverse a genetic mutation with adverse phenotypes. Work is ongoing to improve the frequency of the repair events directed by these novel vectors.

Significance

The use of oligonucleotides without sequence restriction to direct nucleotide exchange in a chromosomal gene is a powerful application for function genomics and gene therapy. While the success rate in mammalian cells continues to grow, it is clear that these approaches will attain a scientific robustness only when the mechanism of base conversion is understood. In this report, we demonstrate that a novel class of oligonucleotide vectors containing locked nucleic acid residues can direct the repair of mutations in the model organism *Saccharomyces cerevisiae*. Since LNA-modified vectors are nuclease resistant and exhibit reduced toxicity in mammalian cells, defining their reaction profiles in this unique reaction may accelerate their use in the treatment of human genetic diseases.

Experimental Procedures

Plasmids and Oligonucleotides

Plasmid pAur101Hyg(Rep)eGFP (Panvera) is a derivative of pAur123 Hyg(rep)eGFP containing a hygromycin-eGFP fusion gene. The plasmid contains a mutation in the hygromycin coding sequence at nucleotide 137 (TAT→TAG), resulting in a stop codon [2], and is integrated into yeast strain *LSY678* [29]. Targeting vectors used in these experiments were categorized as follows. One set contained locked nucleic acid DNA residues labeled as Hyg10NT and Hyg10T; these two oligonucleotides are 24-mers with one LNA residue on each terminus and are designed to target either the nontranscribed (NT) or transcribed (T) strands, respectively. Hyg10/NT/3′ (24-mer), which targets the nontranscribed strand, contains one LNA residue on the 3′ end. Hyg10NT* and Hyg10T* are fully complementary to their respective strands in the target. Each vector contains a single LNA residue on each end, and these oligomers are used primarily as controls. Hyg11NT and Hyg12NT are 24-mer oligonucleotides with two and three LNA residues on each end, respectively. Hyg13 is a 50-mer with five LNA residues on each terminus, while Hyg14 is a 74-mer with five LNA residues on each terminus. Hyg15T is a 15-mer with 12 LNA residues targeting the transcribed strand. Hyg5T/pm and Hyg8T/pm are perfectly matched to the transcribed strand, positioned 5′ and 3′ off-center from the targeted base, respectively. The second types of oligonucleotide vectors are 70-mers (Kan70T), 74-mers (Hyg3S/74T), or 25-mers (Hyg3S/25NT) containing three phosphorothioate linkages on each terminus and are described in Liu et al. [2]. All oligonucleotides containing LNA residues were obtained from Proligo (Boulder, CO), while the Hyg3S series was synthesized by Integrated DNA Technologies (IDT, Coralville, IA).

In Vivo Gene Repair in Yeast

The *Saccharomyces cerevisiae* strain *LSY678* (*mata leu2-3, 112 trp 1-1 ura 3-1 his 3-11, 15 ade 2-1 can 1-100*) was integrated with pAur101Hyg(rep)eGFP by the method of Hashida-Okado et al. [35]. Cells were cultured in YPD medium overnight at 30°C and then diluted into 40 ml YPD medium containing 0.25 μg/ml aureobasidin A. At a density of 2×10^8 cells/ml, the cells were washed twice with 25 ml dH_2O and once with 1 M sorbitol, then resuspended in 120 μl 1 M sorbitol. Forty microliter aliquots were incubated on ice for 5 min with the indicated amount of single-stranded oligonucleotide vector or a combination of two vectors and then electroporated in a 0.2 cm cuvette using a Bio-Rad Gene Pulser apparatus. Electroporation conditions were set at 1.5 kV, 25 μF, 200Ω, 1 pulse, 5 s per pulse length. Following electroporation, the cells were resuspended in 3 ml YPD medium supplemented with 2 M sorbitol and aureobasidin A (0.25 μg/ml) and incubated for 16 hr in a 30°C shaker set at 300 rpm. Aliquots (200 μl) of cells were spread both on YPD containing hygromycin (300 μg/ml) and (200 μl of 10^5 dilution) on YPD containing aureobasidin (0.5μg/ml). The plates were incubated for 3 days at 30°C, and colony growth was quantitated as a function of hygromycin resistance by using an Accucount 1000 (BioLogics, Inc., Gainesville, VA).

Colony PCR for DNA Sequence Analyses

Single colonies were picked from hygromycin-resistant plates and suspended in 50 μl of PCR mix [1× PCR buffer, 200 nM primer

pAUR123F (5′-TCTGCACAATATTTCAAGC), 200 nM primer Hyg1560R (5′-AAATCAGCCATGTAGTG), 500 μM dNTP mix, and 3.5 U Taq polymerase]. PCR conditions were set at 92°C for 2 min, 30 cycles of 92°C for 10s, 52°C for 30s, and 68°C for 1 min, followed by elongation at 68°C for 7 min. Samples were cooled at 4°C and the product band (520 bp) was visualized after electrophoresis on a 1.5% agarose gel. The samples were then purified using a Qiagen PCR purification kit and loaded onto an automatic ABI 310 capillary sequencer for DNA sequence analysis.

D-loop and Double-D-Loop Formation

Formation of joint molecules was carried out in two or three steps, depending on which complex was being formed. A 10 min preincubation with 40 nM oligonucleotides 25 or 74 base pairs in length (^{32}P-labeled for D-loop formation) and 1.5 μM *E. coli* RecA protein in a solution (20 μl) containing 1 mM ATP-γ-s, 25 mM Tris-OAc (pH 7.5), 1 mM $Mg(OAc)_2$, and 1 mM dithiothreitol was incubated at 37°C. The strand exchange reaction was then carried out in the presence of 10 nM pAURHyg(rep) plasmid and an additional 9 mM $Mg(OAc)_2$ for 5 min at 37°C (molar ratio of plasmid to incoming oligonucleotide of 4:1). For double-D-loop formation, an amount of 320 nM annealing [^{32}P]oligonucleotide was added to the complex and incubated for another 10 min at 37°C. After stopping the reaction in dry ice, the joint molecules were deproteinated by the addition of 1% SDS, and associated protein was removed by adding KCl (4°C) to a 100 mM concentration and spinning the solution at 8000 rpm for 5 min at 4°C. The presence of joint molecules was confirmed by 1% agarose gel electrophoresis (1× Tris-borate-EDTA, 97 V, 1.5 hr). Gels were dried on Whatman DE81 filter paper using a Gel Dryer Vacuum System (FisherBioTech) for 2.5 hr at 80°C and visualized by using a Typhoon 8600 PhosphorImager (Molecular Dynamics Inc., CA). Quantification of joint complexes was done using ImageQuant 5.2 software.

Acknowledgments

We thank members of our laboratory, particularly Julia Engstrom, for excellent technical assistance and Dr. Anja van Brabant for helpful discussions. This work was sponsored by NIH grants CA89325-02 and DK56134 and NaPro Biotherapeutics.

Received: May 22, 2002
Revised: July 10, 2002
Accepted: August 22, 2002

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