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Recombination Induced by Triple-Helix-Targeted DNA Damage in Mammalian Cells

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Gene therapy has been hindered by the low frequency of homologous recombination in mammalian cells. To stimulate recombination, we investigated the use of triple-helix-forming oligonucleotides (TFOs) to target DNA damage to a selected site within cells. By treating cells with TFOs linked to psoralen, recombination was induced within a simian virus 40 vector carrying two mutant copies of the *supF* tRNA reporter gene. Gene conversion events, as well as mutations at the target site, were also observed. The variety of products suggests that multiple cellular pathways can act on the targeted damage, and data showing that the triple helix can influence these pathways are presented. The ability to specifically induce recombination or gene conversion within mammalian cells by using TFOs may provide a new research tool and may eventually lead to novel applications in gene therapy.

Gene targeting has been used to introduce foreign DNA into the genomes of mammalian cells through site-specific homologous recombination. By targeting specific genes in embryonic stem cells, it is now possible to study the role of individual genes in laboratory animals (27). However, the frequency of targeted recombination in mammalian cells is low, and the ratio of homologous to nonhomologous integration events is often unfavorable (15, 44). This low frequency of homologous recombination limits the use of this technology for the purpose of gene therapy, and therefore efforts have been made to improve the efficiency of gene targeting.

Previous studies have indicated that a double-strand break (DSB) within the region of homology in the donor molecule can enhance the recombination and targeting frequency (16, 22). However, this increase is modest, and the DSB is likely to result in an increase in nonhomologous recombination as well. Recently, efforts have also been directed toward modification of the recipient site to create a substrate prone to homologous recombination. It has been shown that a site-specific endonuclease, I-*Sce*I, can induce DSBs within extrachromosomal and genomic DNA designed to carry the rare 18-bp recognition site. This strategy has been used to induce intermolecular recombination in both *Xenopus* oocytes (40) and mammalian cells (5, 37). However, this strategy has limited practical application, as it involves the prior introduction of the recognition site within the genome.

Besides DSBs, other types of DNA damage have been shown to be recombinogenic. These include DNA damage from UV radiation (45), chemical carcinogens (52), and photoreactive molecules such as psoralen (38). Psoralens are DNA-damaging agents that intercalate into DNA and form covalent monoadducts (MAs) and interstrand cross-links (XLs) upon exposure to near-UV light (UVA). Photo-induced psoralen interstrand XLs, and to a lesser extent MAs, can induce recombination in bacterial (7, 25), yeast (38), and mammalian (48) cells.

In these studies of psoralen-induced recombination, however, there was no specificity to the distribution of psoralen adducts other than that derived from the propensity of psoralen to intercalate into and cross-link certain common sequences, such as 5′ TpA 3′ sites (6). In contrast, we and others have demonstrated that psoralen damage can be introduced into DNA in a site-specific manner by linking psoralen to an oligonucleotide that is designed to form a triple helix (14, 17, 18, 50). Triplex DNA can be formed when oligonucleotides bind in the major groove of the double helix in a sequence-dependent manner at polypurine/polypyrimidine stretches in duplex DNA (1, 8, 23, 29, 33). The specificity of oligonucleotide-mediated triplex formation has also been used to inhibit transcription and to cleave DNA at unique sites (12, 20, 26, 31, 32, 43).

Using this strategy, we found that triple-helix-targeted psoralen damage can generate site-specific mutations within viral genomes replicating in monkey COS cells (50). Experiments in which COS cells were treated with psoralen-linked triplex-forming oligonucleotides (TFOs), followed by photoactivation of the psoralen, demonstrated that TFOs could enter cells, bind as third strands to duplex DNA, and deliver psoralen adducts in a specific manner, leading to targeted mutations. We also found that high-affinity third-strand binding, itself, could induce mutations in the target gene, via a repair-dependent pathway (51).

In this work, we have investigated the possibility that third strand-targeted psoralen adducts might also be capable of provoking homologous recombination at selected sites within cells. Using a simian virus 40 (SV40)-based shuttle vector carrying two mutant copies of the *supF* reporter gene, we have tested the ability of psoralen-conjugated TFOs to enter cells, to find and bind to their target site, and to stimulate intramolecular recombination within an extrachromosomal substrate. We report here the efficient and specific induction of recombination and gene conversion, as well as mutation, mediated by triple-helix-directed DNA damage within mammalian cells. By comparing a series of psoralen-linked oligonucleotides, we

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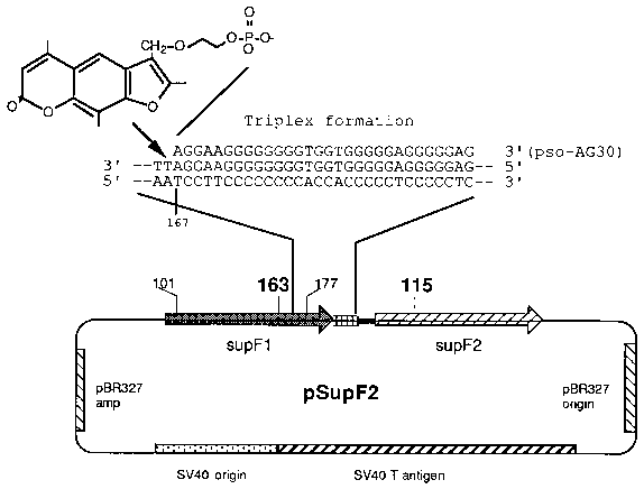

FIG. 1. Schematic representation of the pSupF2 vector. The SV40-based shuttle vector contains two mutant *supF* genes in the form of a tandem dimer. The upstream mutant *supF* gene, *supF1*, contains a C-to-G point mutation at nucleotide position 163; the downstream mutant *supF* gene, *supF2*, contains a G-to-A point mutation at nucleotide position 115. The two *supF* genes are separated by a 9-bp sequence that contains an *Eag*I restriction site used for cloning. At the 3′ end of *supF1* is an engineered polypurine sequence (bp 167 to 196), creating a high-affinity triplex binding site (50). A purine-rich oligonucleotide of 30 nucleotides (pso-AG30) was designed to form a triple helix in the antiparallel triplex motif at this site, as shown previously (1, 32, 50). The oligonucleotide was conjugated at its 5′ end to 4′-hydroxymethyl-4,5′,8-trimethylpsoralen via the 4′ hydroxymethyl position through a two-carbon linker arm. By formation of the triple helix, psoralen intercalation is targeted to the duplex-triplex junction at bp 166 and 167 of *supF1*. Upon photoactivation with UVA, both MAs and interstrand XLs are generated at the thymidines in these base pairs (13).

show that the stimulation of recombination occurs in a sequence-dependent manner. By manipulating the nature of the targeted psoralen damage and by comparing the resulting distribution of nonparental molecules, we provide evidence for multiple pathways of recombination and repair. In particular, targeted psoralen MAs and XLs were found to yield different recombination products. Furthermore, in experiments in which the psoralen damage was delivered by a detachable TFO, we found that the recombination pattern could be influenced by the presence of the third strand itself.

MATERIALS AND METHODS

Vectors. Plasmid pSupF2 was constructed to contain two mutant *supF* tRNA genes in tandem (Fig. 1). The *supF* genes carrying different point mutations were isolated in the course of mutagenesis experiments, either in the shuttle vector pSP189 (30) or in the vector pSupFG1 (50). pSupFG1 carries a novel *supF* gene modified at several positions to create a polypurine binding site for high-affinity triple-helix formation. The pSupFG1 derivative, pGW47, containing a C-to-G mutation at bp 163 within the *supFG1* gene (yielding the gene designated here *supF1*), was digested with *Eag*I, and the mutant *supF* gene in the pSP189 derivative, carrying a G-to-A mutation at nucleotide position 115 (designated *supF2*), was amplified by PCR using primers containing *Eag*I linkers (5′ ACACGGCC GTACCTGTGGTGGGGT 3′ [forward] and 5′ ACACGGCCGTGGTGGTGG GGGAAG 3′ [reverse]). The resulting 100-bp fragment was subcloned into the *Eag*I-digested pSupFG1 DNA to give the pSupF2 shuttle vector containing the two mutant *supF* genes as direct repeats separated by 9 bp. The orientation of the cloned fragment was confirmed by DNA sequencing. The vector also contains the SV40 origin of replication and large-T-antigen coding sequence along with the β-lactamase gene and replication origin from pBR327, as described previously (50).

Oligonucleotides. Psoralen-linked oligonucleotides were supplied by Oncor-Pharm (Gaithersburg, Md.). The psoralen is incorporated into the oligonucleotide synthesis as a psoralen phosphoramidite, resulting in an oligonucleotide linked at its 5′ end via a two-carbon linker arm to 4′-hydroxymethyl-4,5′,8-trimethylpsoralen. The oligonucleotides used in this study were pso-AG30, (5′ psoralen-AGGAAGGGGGGGGTGGTGGGGGAGGGGGAG-3′), pso-AG10 (5′ psoralen-AGGAAGGGGG 3′), and pso-Mix30 (5′ psoralen-AGTCAGTCA GTCAGTCAGTCAGTCAGTCAG 3′). The psoralen-conjugated oligonucleotides were also synthesized to contain a 3′ propylamine group to minimize susceptibility to degradation by 3′ exonucleases (20).

In vivo targeting and recombination assay. Monkey COS-7 cells, obtained from the American Type Culture Collection (1651-CRL), were grown in Dulbecco's modified Eagle's medium (GIBCO/BRL, Bethesda, Md.) supplemented with 10% fetal calf serum (Sigma, St. Louis, Mo.). At 80% confluency, the cells were detached by trypsinization and washed three times in growth medium. The cells were resuspended at a density of 10^7/ml, and plasmid DNA was added at 3 μg of DNA per 10^6 cells. The cell-DNA mixture was then incubated on ice for 10 min. Transfection of the cells was carried out by electroporation using a Bio-Rad gene pulser at a setting of 25 μF/250 W/250 V in 0.4-cm transfection cuvettes.

Following electroporation, the cells were kept on ice for 10 min and then transferred to 37°C for 30 min. The cells were diluted with growth medium and exposed to the psoralen-linked oligonucleotides added to the medium at a concentration of 2 μM. Concentrations of less than 1 μM were ineffective, whereas a concentration of 10 μM was toxic to the cells (data not shown). The suspension samples were incubated at 37°C with gentle agitation every 15 min. After 2 h, the cells were exposed to UVA light at a dose of 1.8 J/cm^2. A protocol in which the TFO addition was performed 12 h after vector transfection has yielded evidence of gene targeting (50). However, prior trypsinization and electroporation appears to enhance subsequent cellular uptake of the TFOs, although this has not been fully optimized.

Irradiation of samples with UVA was performed with 365-nm lamps supplied by Southern New England Ultraviolet Co. (Branford, Conn.). A radiometer (International Light, Newburyport, Mass.) was used to measure the lamp output (typical UVA irradiance of 5 to 7 mW/cm^2 at 320 to 400 nm). A window glass filter was used to eliminate any UVB contamination during the UVA irradiation. All samples, including control cells not exposed to oligonucleotides, received UVA irradiation. Following UVA treatment, the cells were distributed into tissue culture dishes at a density of 10^5 cells per cm^2.

Shuttle vector isolation and analysis. Forty-eight hours after UVA irradiation, the cells were harvested for plasmid DNA isolation by using a modified alkaline lysis procedure (50), and the resulting vector DNA was subjected to digestion with *Dpn*I (to eliminate any unreplicated molecules that had not acquired the mammalian methylation pattern) and used to transform bacteria for genetic analysis of *supF* gene function exactly as previously described (50). Selected colonies were purified, and the plasmids were isolated for either PCR or DNA sequence analysis.

In vitro triplex binding and photoadduct formation. In a reaction volume of 10 μl, 3 μg (50 nM) of the pSupF2 DNA (or pGW47 vector DNA, as indicated) was incubated with a 100:1 molar excess of pso-AG30 (5 μM) for 2 h at 37°C in 10% sucrose, 20 mM $MgCl_2$–10 mM Tris (pH 8.0)–1 mM spermidine (triplex binding buffer). Irradiation of samples with either UVA or visible light was performed as previously described (13, 17, 34, 49). Transfection of the vector DNA by electroporation and shuttle vector rescue and analysis were as above except that the cells were distributed to culture dishes immediately after electroporation.

To create a site-specific psoralen adduct in the absence of an associated oligonucleotide, we generated targeted psoralen adducts in vitro, as above, using a TFO synthesized to contain a disulfide linker between the tethered psoralen and the nucleic acid portion, pso-S-S-AG13. The design and synthesis of this reagent have been described elsewhere (34, 49). Following triplex formation and UVA irradiation, the oligonucleotide was detached by reduction with dithiothreitol (DTT) as described previously (34, 49).

Product analysis. Screening of the recombinants by PCR amplification of the sequences containing the *supF* gene(s) in the vectors was performed with the following primers: 5′ GGCGACACGGAAATGTTGAA 3′ (forward) and 5′ TTAGCTTTCGCTAAGGATCCGG 3′ (reverse). DNA sequencing was carried out as described previously (50). The sequencing primer was the same as the forward primer used for screening of the recombinants (see above).

RESULTS

Design of the vector to detect induced recombination. The vector, pSupF2, was constructed to test the ability of triplex-directed DNA damage to trigger recombination between tandem *supF* genes flanking the third-strand binding site (Fig. 1). *supF* encodes an amber suppressor tyrosine tRNA whose function can be screened by a colorimetric assay in host bacteria carrying a *lacZ* nonsense mutation. To facilitate the detection of recombinants via a phenotypic screen, we inactivated the upstream *supF1* gene in the vector with a C-to-G mutation at bp 163 (163 mutation), whereas the downstream *supF2* gene was inactivated with a G-to-A mutation at position 115 (115 mutation). Hence, recombination between *supF1* and *supF2* has the potential to reconstruct a fully functional *supF* gene,

which can be identified by the resulting dark blue colonies on indicator plates.

In addition, we were able to determine assay conditions in which the parental pSupF2 vector yields a faint blue colony color. Although we initially thought that the 163 mutation would completely inactivate the *supF1* gene, we found that this allele actually retained a low level of suppressor activity. This finding allowed us to set up the assay to identify additional nonparental plasmids resulting from events (including not only recombination but also mutation) that completely eliminate or at least further diminish suppressor function. Such plasmids are recognized by the resulting pure white colonies.

Because of the modifications made to create the *supFG1* gene from which *supF1* was derived, recombinational events between *supF1* and *supF2* can be characterized at the sequence level not only by their effects on the 115 and 163 mutations but also by their effects on 3 noninactivating polymorphic mutations that distinguish the two genes. *supF1* differs from *supF2* by mutations at positions 101 (T:A to G:C) and 177 (A:T to C:G) and by a 12-bp insertion between bp 183 and 184 (50). These changes do not disrupt suppressor function but were introduced to create the high-affinity triple-helix binding site embedded within the 3′ end of the gene. Specifically, the mutation at position 177 was made to eliminate the interruption in the existing polypurine/polypyrimidine run in *supF* at that position. This change was accompanied by a compensatory change at position 101 to maintain base pairing in the amino acid acceptor stem of the tRNA (50). The 12-bp homopurine/homopyrimidine insertion at the 3′ end of the *supF1* gene (just beyond the sequences coding for the mature tRNA) extends the length of the polypurine/polypyrimidine run in the gene to 30 bp (Fig. 1).

A polypurine oligonucleotide conjugated to psoralen at its 5′ end, pso-AG30, was designed to bind to the 30-bp polypurine/polypyrimidine site in the vector and position the tethered psoralen for intercalation and adduct formation at bp pairs 166 and 167 of the *supF1* gene (Fig. 1). The binding of pso-AG30 to this site was determined to have an equilibrium dissociation constant (K_d) of 3×10^{-9} M, and this binding was previously found to be sufficient for intracellular triplex formation and targeted mutagenesis in a shuttle vector carrying the *supFG1* gene (50).

Novel products induced by triplex-directed psoralen adducts in COS cells. Using the pSupF2 vector, we examined whether a series of psoralen-linked oligonucleotides could stimulate recombination via intracellular triple-helix formation. In these experiments, pSupF2 DNA was first transfected into the cells by electroporation. The cells were extensively washed, and the oligonucleotides were added to the growth medium of the cells in suspension. After 2 h to allow for entry of the oligonucleotides into the cells and for third-strand binding, the cells were irradiated with UVA to photoactivate the psoralen. Control cells received no oligonucleotide but were still irradiated with UVA. The treated cells were transferred to culture dishes for 48 h before rescue of the vector DNA for analysis.

Colonies were identified by their nonparental phenotype, either blue or pure white (Table 1). Cells not exposed to any oligonucleotide gave background frequencies of 0.02% blue colonies and 0.03% white colonies. When cells were treated with pso-AG30, blue colonies were generated at a frequency of 0.49%, 25-fold above the background. Pso-AG30 treatment of the COS cells also produced 0.86% nonparental pure white colonies, compared to 0.03% in the absence of oligonucleotide treatment. Pso-AG10, which can bind with specificity in vitro to the pSupF2 vector but has a 270-fold-lower binding affinity for triplex formation (50), did not induce recombinants above the background level. Hence, the binding of this TFO, although specific, is too weak to mediate intracellular targeting events. Treatment of the cells with pso-Mix30, which consists of a nonspecific mixed sequence and which is unable to bind as a third strand to the vector, yielded only background levels of recombination.

TABLE 1. Recombination induced by triple-helix-targeted DNA damage in COS cells

Oligonucleotide	K_d for third-strand binding[a] (M)	Blue colonies (%)[b]	White colonies (%)[b]
None	NA	0.02 (5/23,797)	0.03 (4/13,136)
Pso-Mix30	ND	0.01 (2/16,410)	0.03 (5/16,410)
Pso-AG10	8×10^{-7}	0.01 (1/14,012)	0.03 (4/14,012)
Pso-AG30	3×10^{-9}	0.49 (102/20,751)	0.86 (148/17,251)

[a] NA, not applicable; ND, not detectable.
[b] The values represent the frequencies of nonparental colonies produced by pSupF2 shuttle vector plasmids rescued from COS cells that were treated with oligonucleotides as indicated.

Analysis of products. To ascertain the nature of the pso-AG30-induced events, we used PCR analysis to determine the *supF* gene copy number in the nonparental plasmids (Fig. 2). Plasmid DNA from 60 blue colonies was amplified by using primers flanking the tandem *supF* genes in pSupF2. All 60 gave an amplified band of 420 bp, consistent with the presence of both copies of the *supF* gene (an example is shown in lane 4). This matches the band obtained from amplification of the parent plasmid, pSupF2 (lane 1). Hence, all of the blue recombinants tested appear to have arisen from conservative events in which information transfer has taken place to generate at least one functional *supF* gene without the loss of vector sequences.

Similarly, 400 pure white colonies were tested, and 90.5% also yielded a band identical to that obtained from the parental pSupF2 plasmid (one example is shown in lane 5), indicating, again, the presence of both *supF* genes. From the other 9.5% of the white colonies, however, a smaller amplified fragment was obtained (lane 6); this fragment matched the size of the

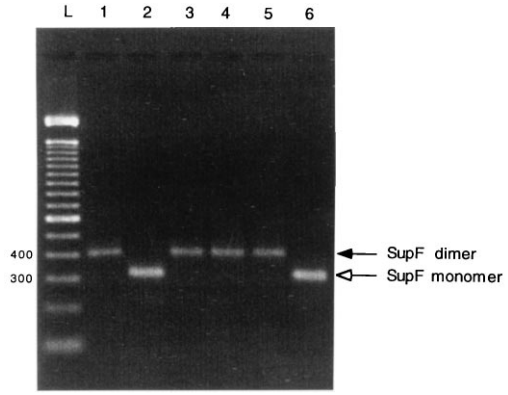

FIG. 2. PCR analysis of the structures of the recombination products. Lanes 1 and 2 are control lanes showing the amplification patterns obtained from the parental vector pSupF2 containing the two tandem *supF* genes and from the vector pSupFG1 containing just a single *supF* gene, respectively. Lane 3 shows the amplified fragment from a parental faint blue colony. Lane 4 represents a nonparental blue colony, while lanes 5 and 6 show amplified fragments obtained from selected nonparental white colonies. Lane L includes a 100-bp ladder to allow fragment size estimation.

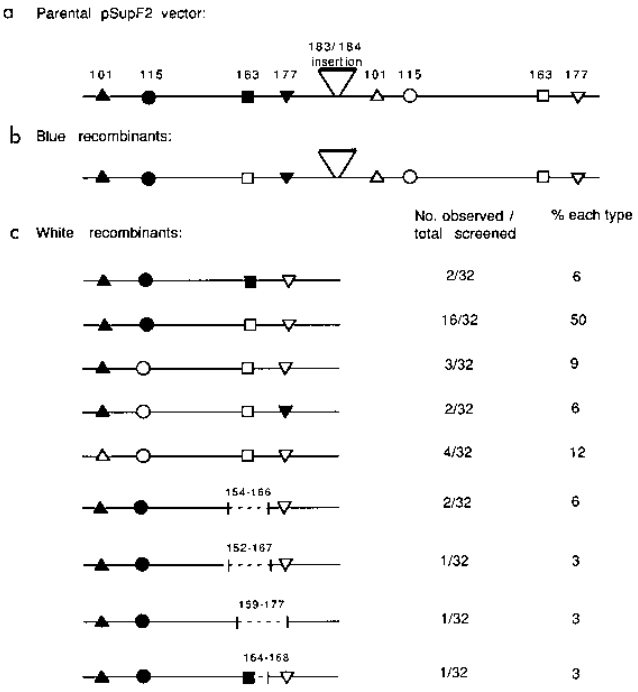

FIG. 3. Schematic illustration of the nonparental products induced by triplex-targeted DNA damage in COS cells as determined by DNA sequence analysis. (a) The parental pSupF2 vector containing the *supF1* and *supF2* genes. (b) The pattern of the nonparental blue products. (c) The nonparental white recombinants. For each type of white recombinant, the number observed is listed. The diagrams show the recombination patterns with regard to the mutations at bp 163 and 115, along with the 101 and 177 polymorphisms and the 12-bp polypurine/polypyrimidine insertion that is part of the triplex binding site. Sequences from the upstream gene (filled symbols) and those from the downstream gene (open symbols) are depicted.

310-bp fragment from the pSupFG1 vector (50), which contains only a single *supF* gene (lane 2). This result suggests that at least some of the nonparental products are produced in nonconservative recombination events, involving the loss of sequences.

To further examine the recombinant vectors, we carried out DNA sequence analysis of the *supF* gene region (Fig. 3 and 4). All 20 blue recombinants sequenced were found to have the same pattern, with both *supF* genes present and with the 163 mutation in the upstream *supF1* gene corrected to the wild-type sequence. This C:G-to-G:C change at position 163 restores full function to the upstream gene, resulting in the blue colony phenotype. This product may result from a recombinational process involving nonreciprocal exchange of information

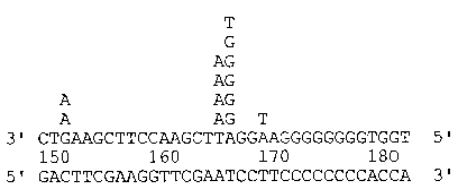

FIG. 4. Sequences of the mutations induced by triple-helix-targeted psoralen adducts in the pSupF2 vector. The mutations were clustered at and around the targeted psoralen intercalation site at bp 166 and 167 of the upstream *supF1* gene. Base pairs 150 to 183 of this gene are shown, with the mutations listed indicating a change from the nucleotide in the upper strand.

from the downstream *supF2* gene to the upstream *supF1* gene, a process characteristic of gene conversion.

Sequence analysis of the pure white colonies revealed a variety of products (Fig. 3 and 4). Those judged by PCR to have arisen from nonconservative events were found to be recombinants carrying single mutant copies of the *supF* gene, with different mixtures of markers from the upstream and downstream genes. Most of these single-gene recombinants had sequences consistent with single crossover events, with the relative frequencies roughly proportional to the distances between markers (Fig. 3). Sixteen of 32 of the recombinant genes appeared to arise from a crossover between bp 115 and 163. Less frequent single crossovers were found within other regions. Two genes were found that consisted of *supF1* sequences at the 5′ and 3′ ends and *supF2* sequences in the middle, suggesting possible triple crossover events, considering that a third crossover is necessary to connect the 3′ end of *supF1* to the downstream plasmid sequences. Several putative recombinants were also found to contain single *supF* genes with short deletions encompassing the targeted psoralen cross-linking site.

Most of the white colonies, however, were determined by PCR to carry vectors retaining both *supF* genes. Sequence analysis of 14 of these revealed that all had undergone mutation rather than recombination or gene conversion (Fig. 4). The mutations were found predominantly at bp 166 and 167 of the *supF1* gene, precisely at the targeted psoralen intercalation site. These mutations resulted in pure white colonies because they eliminated the residual suppressor activity of the *supF1* gene. This analysis does not rule out the possibility that a small proportion of the nonparental white colonies arose from conservative gene conversion events, such as an event in which bp 115 in the upstream gene is converted to the sequence of the downstream gene. However, such products appear to be rare and would be identified only by an extensive DNA sequencing effort.

Therefore, we can estimate that of the total of 0.86% nonparental white colonies produced by pso-AG30, approximately 0.77% are point mutations and 0.09% are single-gene recombinants, including 0.014% consisting of recombinants with additional small deletions. Overall, in the intracellular targeting experiments, detectable recombinants (blue and white) were induced at a frequency of 0.58% and mutations were generated at a frequency of 0.77%.

Blue recombinants are not explained by reversion. Interpretation of the blue colonies as arising from recombination or gene conversion, however, is potentially complicated by the possibility that they may instead originate from simple reversion mutation of bp 163 in the upstream *supF1* gene to the wild-type sequence. This mechanism was of particular concern because point mutations were found at a high frequency among the nonparental white colonies. To test this, we measured the induced reversion frequency of the *supF1* gene carried as a single *supF* gene in a shuttle vector otherwise identical to pSupF2, pGW47 (50). In this experiment, triplex formation with pso-AG30 and UVA photoactivation were carried out both in vivo and in vitro, the latter to maximize the extent of vector modification (17). We observed blue colonies at frequencies of only 0.06% (2/3,100; in vivo) and 0.007% (2/27,562; in vitro). Therefore, reversion mutation cannot explain the 0.49% blue colonies produced in the pSupF2 experiments, and so they must arise from a nonmutational event, such as gene conversion.

Comparison of targeted MAs and XLs. To elucidate the pathways leading to the different classes of recombinants, we took advantage of the ability to experimentally manipulate the

TABLE 2. Influence of the nature of the targeted psoralen adduct and of the presence of the third strand on the recombination products

Oligonucleotide	Irradiation wavelength (nm)	Blue colonies (%)	White colonies (%)
None	447	0.01 (2/18,244)	0.02 (4/18,244)
	365	0.02 (2/11,790)	0.02 (2/11,790)
Pso-AG30	447[a]	0.01 (2/20,034)	0.47 (95/20,034)
	365[a]	1.23 (150/12,160)	13.62 (1,220/8,960)
Pso-S-S-AG13 + DTT[b]	365[a]	0.03 (4/12,235)	3.46 (423/12,235)

[a] Irradiation with 447-nm visible light generates only psoralen MAs, while 365-nm UVA irradiation induces mostly XLs (13).

[b] Following triplex formation and photoactivation with pso-S-S-AG13, reduction of the disulfide bond with DTT detaches the oligonucleotide portion and leaves an isolated psoralen adduct without an associated third strand (34).

types of psoralen photoadducts in the context of the triple helix. By using light of different wavelengths for photoactivation, the ratio of XLs and MAs can be controlled (13). To most efficiently probe cellular pathways, we used a protocol in which the triplex-targeted damage was introduced into the pSupF2 vector in vitro, followed by transfection of the vector DNA into COS cells and subsequent rescue of the products and analysis in bacteria, as above. This in vitro protocol allows for more efficient triplex formation and for more precise control over photoadduct distribution. We used doses of UVA and visible light for which we had previously characterized the photoadduct distribution: (i) 1.8 J of UVA (365 nm) per cm^2, producing 65% XLs between the T's at the duplex triplex junction at bp 166 and 167, 20% MAs on the T in 166, and 10% MAs on the T in bp 167; and (ii) 12 J of 447-nm visible light per cm^2, which results in photomodification of 40% of the vector molecules with MAs on the T in bp 166, less than 1% MAs on the T in bp 167, and less than 1% XLs (13).

Overall, fewer nonparental products were induced following visible light photoactivation than after UVA (Table 2). This is not surprising, since the dose of visible light used leads to significantly less photomodification compared to the UVA dose. More importantly, the types of nonparental products differed depending on the wavelength used for photoactivation, with a white-to-blue ratio of 47:1 for visible light versus 11:1 for UVA. In fact, essentially no blue recombinants were produced over the background frequency with visible light. These results suggest that the blue recombinants most likely arise from the targeted XLs. (It cannot be completely ruled out that some blue recombinants come from the MAs on the 167 T, but this photoproduct is uncommon.) Analysis of the white colonies induced by pso-AG30 and visible light revealed that 15% (3/20) carried single recombinant *supF* genes, a frequency similar to the 9.5% seen with UVA. All three of the recombinants had crossovers between bp 115 and 163, matching the most common recombinant induced in conjunction with UVA. The other white colonies had vectors retaining both *supF* genes. Although we did not sequence these products, they are likely to be mutations, since we have shown in previous work that triplex-targeted MAs in *supF* can generate T:A-to-A:T transversions at bp 166 as a consequence of translesion synthesis during replication (34, 49).

Comparison of data for pso-AG30 in Tables 1 and 2 suggests that a higher proportion of blue colonies are produced when third-strand binding and photoproduct formation occur in vivo as opposed to in vitro. It is possible that the photoproduct distribution may be different in the two cases. Also, since multiple repair and recombination pathways may act on the vector substrate, there may be differences in the metabolism of the vector DNA depending on whether the targeted damage is introduced before or after transfection.

Influence of the third strand on the recombination products. We had previously found that an associated third strand can partially inhibit the repair of a targeted psoralen adduct by blocking repair-related endonuclease activity (49). This was seen to cause a change in the spectrum of psoralen-induced mutations. We therefore hypothesized that the presence of the triple helix might also influence the pattern of recombination products. To test this, we used a psoralen-conjugated oligonucleotide, pso-S-S-AG13, designed to bind to the polypurine site at the 3′ end of the *supF1* gene in pSupF2 and synthesized to contain a disulfide linker between the psoralen and the oligonucleotide portion. Following triplex formation and UVA photoactivation, the vector DNA sample was treated with DTT to reduce the disulfide bond within pso-S-S-AG13 and to thereby detach the oligonucleotide from the psoralen adduct (34, 49). The resulting vector DNA, containing an isolated adduct in the absence of an associated third strand, was transfected into COS cells, and recombination was assayed as above. The rescued products were notable for the absence of any blue recombinants above the background level (only 0.03% [Table 2]) in spite of a reasonably high frequency of nonparental white colonies (3.5%). This pattern is clearly different from that induced by the nondetachable pso-AG30 in vitro (1.2% blue and 13.6% white; Table 2). Hence, the production of blue recombinants is enhanced by the presence of the third strand in conjunction with a psoralen XL.

The white colonies in this experiment were determined to be similar to those induced by pso-AG30. By PCR, 15% (3/20) were monomers, a proportion similar to that seen with pso-AG30 in the previous analysis (9.5%). By DNA sequence analysis, two of these three had single crossovers between bp 115 and 163 and one had a crossover between bp 163 and 177.

DISCUSSION

We have shown that TFOs can target DNA damage and induce site-specific genetic changes within mammalian cells. Cells pretransfected with an SV40-based shuttle vector carrying two mutant copies of the *supF* gene were subsequently treated with psoralen-conjugated TFOs, and it was found that such oligomers could enter cells and provoke nonconservative recombination, gene conversion, and mutation within the vector in a sequence-dependent manner. The specificity of the reaction was evident because only the pso-AG30 oligonucleotide, which binds with high affinity to the target site, was found to generate nonparental products in the cells. The other psoralen-conjugated oligonucleotides were not active in the assay, either because they bound too weakly (pso-AG10) or not at all (pso-Mix30).

The third-strand-targeted damage induced a variety of nonparental products, a finding consistent with the presence of multiple competing pathways within the cell poised to metabolize damaged DNA. We were able to detect the various products simultaneously in our assay because of the structure of our vector, which can report both recombination and mutation. In this regard, two observations are particularly noteworthy. The third-strand-targeted damage was found to induce conservative, gene conversion events in the vector. Such events are unusual for this type of extrachromosomal substrate and more characteristic of processes involving chromosomal loci (2). Second, the recombination and gene conversion pattern was found to be influenced by the presence of the triple helix. Without the third strand, gene conversion was not seen. Hence, the TFOs

not only serve as DNA binding ligands to direct DNA damage within the cells but also influence cellular metabolism of the DNA at the binding site.

The observation of mutations, which were generated mainly at the targeted psoralen intercalation site at the duplex/triplex junction in the upstream *supF1* gene, confirms the results of our previous work (50) in which targeted mutations were induced by intracellular triplex formation in a vector carrying a single wild-type *supFG1* gene designed to report only mutations. In that work, mutations were induced by pso-AG30 at a frequency of 2.1%, somewhat higher than but in the same range as the 0.77% mutations reported here. This difference may be attributed to the presence of the second *supF* gene in the pSupF2 vector, which can participate in recombinational repair, thereby reducing the frequency of mutations and leading to other products. Interestingly, the mutations observed here include not only T:A-to-A:T transversions (as seen in our prior work) but also T:A-to-C:G transitions, suggesting that the pattern of mutations may also be influenced by the presence of the second *supF* gene, although the sample size may be too small to allow us to draw firm conclusions. Nonetheless, most of the mutations were at the targeted psoralen intercalation site at bp 166 and 167.

To account for the range of products observed, we propose that the triplex-targeted damage provokes at least two major pathways of DNA metabolism, one of which is nucleotide excision repair (NER). Several lines of evidence suggest that excision repair of psoralen XLs begins with dual incisions on one strand flanking the adduct site (3, 4, 34, 35, 46, 47, 49). This leads to a gap in that strand opposite a still damaged strand containing a psoralen adduct, which in the case of the triplex-targeted lesion is not only cross-linked to the released fragment of the repaired strand but also tethered to the TFO. The gap is then filled in by repair polymerase(s), requiring DNA synthesis across the damaged site. A significant portion of the targeted mutations likely arise from misincorporation during translesion synthesis in this step (34, 49). Another round of excision repair then acts on the other strand, which contains the second half of the adduct.

This repair pathway, however, is complicated by the presence of the triple helix, which presents the cell with a complex lesion that challenges the repair machinery. We previously found in both in vitro and in vivo assays that the third strand can alter repair by blocking endonuclease activity and by influencing strand selection during NER (49). In particular, we found evidence suggesting that in the context of the *supFG1* gene (from which *supF1* was derived), the third strand causes a bias which favors repair of the pyrimidine-rich strand (49).

Therefore, with regard to the pSupF2 vector carrying a triplex-targeted adduct, we propose that NER activity would generate a repair gap predominately in the pyrimidine-rich strand of *supF1* (Fig. 5). Based on the reported patch size for NER (6 nucleotides 3′ and 22 nucleotides 5′ of the lesion) (19), we would predict that this gap would include the 163 but not the 177 position. The gap could then be filled in by repair synthesis, requiring translesion bypass across the psoralen adduct at bp 166 and leading at some frequency to mutations. Of course, in some cases, the lesions are repaired correctly, maintaining the sequence of the parental molecules, but such products are not distinguishable in our assay.

Alternatively, because of the presence of the downstream *supF2* gene, the gapped vector molecule could also undergo a rearrangement in which the undamaged strand of the downstream gene is brought into juxtaposition with the gapped strand of the upstream gene. In this case, gap-filling repair synthesis could then occur on an undamaged template and would not have to bypass an adduct. Therefore, there would be no mutation at bp 166 and 167. However, the sequence of the transferred strand from the downstream gene at position 163 is wild type; therefore, fill-in synthesis would incorporate the wild-type sequence at position 163, accounting for correction and gene conversion of the upstream gene. Since we predict that the repair gap would not extend to position 177, the sequence at that base pair in *supF1* would be unaltered, just as we observed in the blue colonies. This model is consistent with studies of psoralen mutagenesis and repair in *Escherichia coli*, which have shown that recombination can play a role in the repair of XLs and can reduce the frequency of psoralen-induced mutations (3, 41, 42).

In all of the blue colonies analyzed, we observed conversion of the *supF1* and not of the *supF2* gene. This fits the model, because it is the damaged gene that receives information from the undamaged one in the course of repair, and the TFO targets the damage specifically to the *supF1* gene. (A converted downstream *supF2* gene could be detected if it were produced because both the *supF1* and *supF2* genes are expressed from the upstream β-lactamase promoter in the vector.)

The model also accounts for the observation that generation of the blue colonies requires the presence of the third strand in association with the psoralen adduct. In the absence of the triple helix, the proposed strand bias in repair would likely be reversed, since the furan-side adduct in a psoralen XL is usually repaired first (4, 47). In the context of the triplex-targeted psoralen adduct in the *supF* gene, the furan adduct is on the T at position 166 in the purine-rich strand (13). Therefore, the repair gap would likely be created in that strand, and it would extend in the 5′ direction beyond position 177. Recombinational repair in this gap might correct the mutation at bp 163, but it would also change the sequence at bp 177 to that of the downstream gene. In the absence of a compensatory change at bp 101, this would eliminate suppressor function, and the resulting colony would not be blue.

Similarly, the model also explains why no blue colonies were induced above the background level following visible light activation (Table 2). The visible light-induced lesions are almost all MAs on the T at bp 166 in the purine-rich strand (13), and so any excision repair that might take place would occur on that strand, again theoretically leading to conversion of both bp 163 and bp 177. Furthermore, previous work suggests that the targeted monoadducts at bp 166 are actually resistant to excision repair because the triple helix appears to block repair endonuclease activity on the purine strand (49).

Hence, the generation of blue colonies in our assay can be explained by a pathway of lesion-initiated gene conversion. Intrachromosomal gene conversion between direct repeats in mammalian cells has been previously observed by Bollag et al. (2). In work presented here, however, we have triggered gene conversion by using site-directed DNA damage. The fact that we could detect only a single type of nonparental molecule that we can attribute to this pathway reflects the specificity of the third-strand-targeted adduct.

Although this recombinational repair and gene conversion model therefore accounts for much of the data, it does not explain the origin of nonparental white colonies consisting of single-gene recombinants. These products likely arise from another pathway consistent with the single-strand annealing model of Lin et al. (24). The initial steps in this pathway involve the introduction of DSBs into the vector DNA at or around the site of targeted damage. It is well established that psoralen XLs can induce DSBs in eukaryotic cells (9, 21). We do not have direct evidence that such breaks are produced by the more complex psoralen-triple helix lesion in this work.

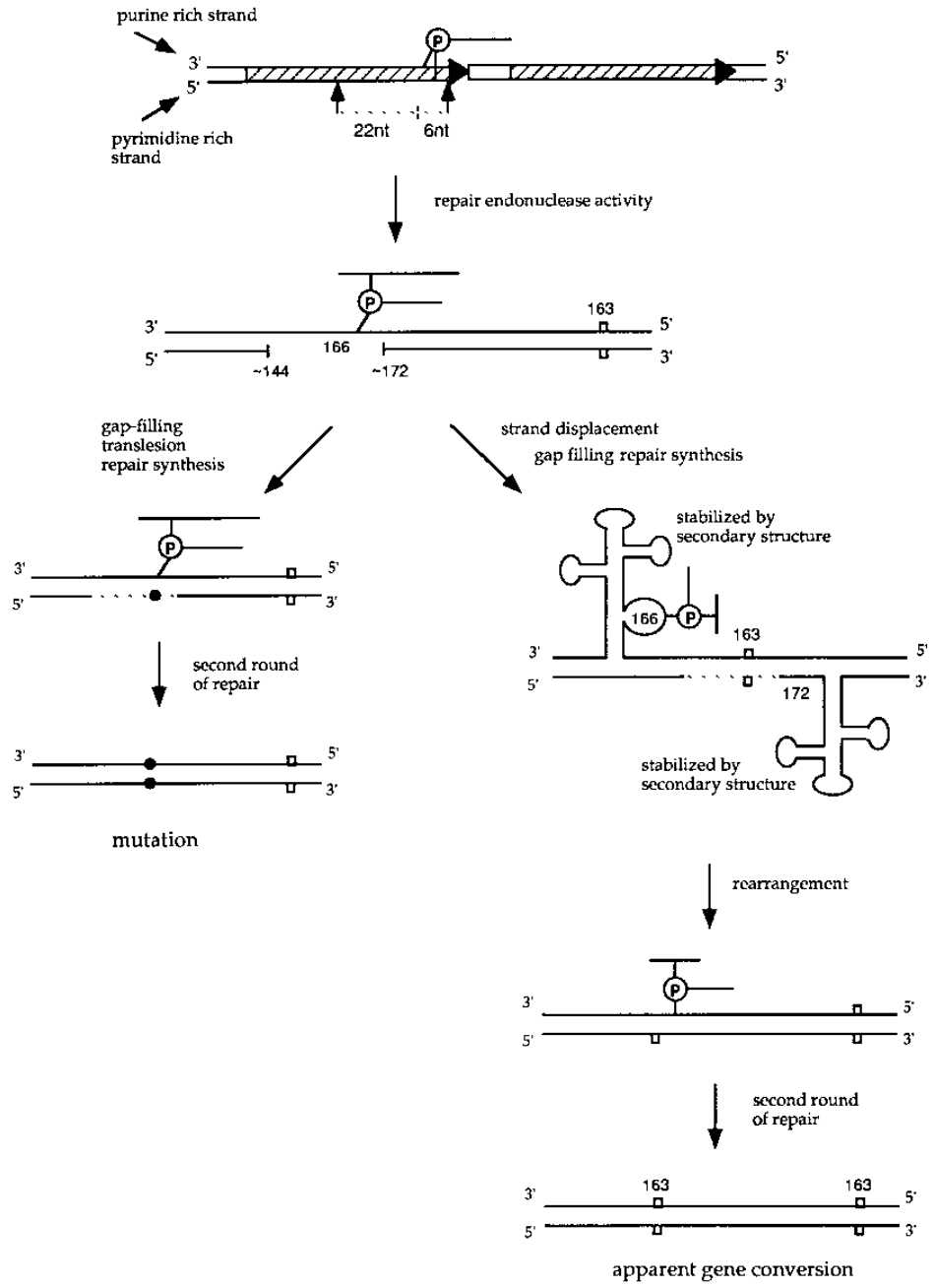

FIG. 5. Model for recombinational repair and gene conversion in pSupF2 induced by triple-helix-targeted psoralen adducts. See text for details. nt, nucleotides.

However, the products are consistent with the model shown in Fig. 6, in which DSBs are the initiating event. These breaks would be followed by exonuclease activity to expose homologous single-stranded regions that could anneal to form heteroduplexes. The formation of such heteroduplexes in extrachromosomal recombination substrates carrying duplicated genes has been demonstrated (10). Resolution of the heteroduplexes in various ways via mismatch repair can then generate the observed recombination products. The mosaic pattern of parental markers detected in the recombinants is similar to other observations of nonconservative recombination products in mammalian cells (11).

In particular, the predicted heteroduplex between the repeated *supF* sequences may contain several mismatches. The exact structure of the heteroduplex depends on the positions of the strand breaks; however, single base pair mismatches at positions 101, 115, 163, and 177, along with the 12-base loop caused by the insertion at bp 183 and 184 in the strand contributed by the upstream *supFG1* gene, are all possible. Theoretically, these could each be repaired in two ways, leading to 32 different products. The fact that we have observed only 6 of the 32 possible products may reflect, in part, the nature of the assay, in which we identify only those recombinants that cause a colony phenotype change. For example, recombinants carrying the 163 mutation (along with the coupled 101 and 177 polymorphisms) would produce a faint blue colony color similar to the parental color and would not be counted.

In addition, the relative distribution of the different recom-

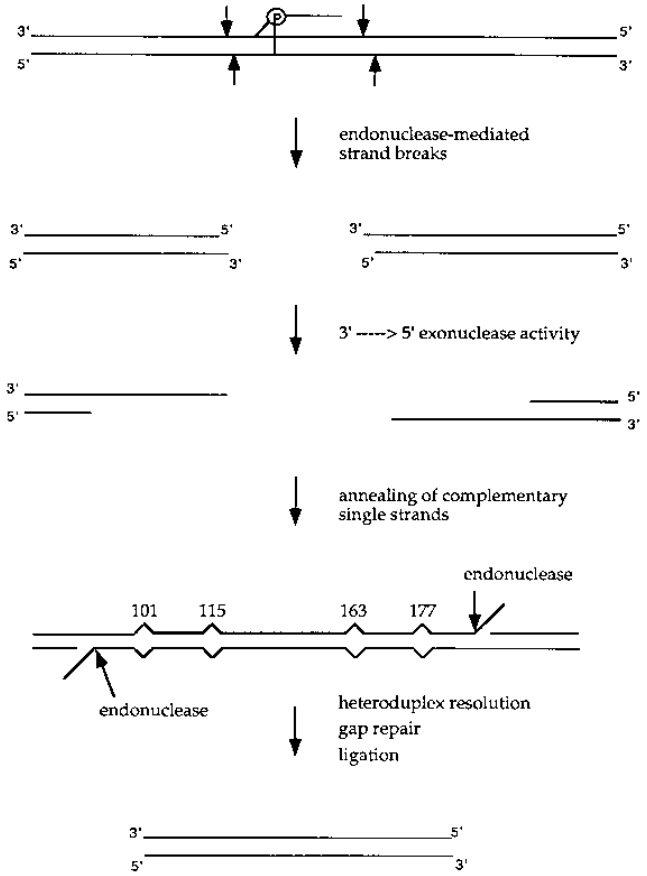

FIG. 6. Model for intramolecular recombination induced by triplex-targeted adducts. The polarity of exonuclease degradation is shown as 3′ to 5′, as in the model of Lin et al. (24), but this polarity is not proven. A 5′-to-3′ polarity is just as plausible and, in fact, has been established in *Xenopus* oocytes (28).

binants correlates with the distances between the markers. The most frequent white recombinant has a sequence in which the 101 and 115 markers are derived from *supF1* and the 163 and 177 markers are from *supF2*, suggesting corepair of the two sets of closely spaced markers in the heteroduplex model, resulting in apparent recombination between positions 115 and 163. The less frequent products show recombination either between 101 and 115 or between 163 and 177. The range of products may also be influenced by sequence-dependent biases in mismatch resolution (10).

If DSBs are made on both sides of the triple helix, we would expect deletion of the polypurine insertion site at the 3′ end of *supF1* and possibly of adjacent sequences. In all of the recombinants analyzed, this site is absent. DSBs in the vicinity of the triplex-delivered damage may also explain the products containing short deletions of *supF* sequences in this region (Fig. 3), since illegitimate and homologous recombination events are known to compete for DNA ends in mammalian cells (36, 37).

Our inability to detect single-gene recombinants giving rise to blue colonies may simply be due to the stringency of the assay. Recombination to generate a functional suppressor gene must occur via a very specific pattern of multiple crossovers. One crossover must take place between the mutations at bp pairs 115 and 163. However, the polymorphisms in the upstream *supF1* gene at positions 101 and 177 (introduced to enhance the triplex binding site) complicate the process, since a functional gene requires the presence of either both changes or neither change. A recombination event correcting either the 115 mutation or the 163 mutation would yield a blue colony only if it also preserved the pairing of the 101 and 177 polymorphisms. This would therefore require a triple crossover event, with crossovers between 115 and 163, between 163 and 177, and between 177 and the end of the *supF1* gene at position 183 (in order to establish continuity with the downstream flanking plasmid sequences). Out of 32 white recombinants sequenced (Fig. 3), we did observe two molecules apparently resulting from triple crossover events. However, the overall frequency of such molecules was 0.006%. If blue colonies were to be produced by triple crossover events of the same frequency, they would constitute 1% or less of the total blue colonies and thus could have been missed in our analysis. In the same way, we may have missed other types of single-gene recombinants with sequences conferring a low level of suppressor activity, yielding faint blue colonies indistinguishable from those produced by the parental vector.

In addition, to produce a detectable recombinant, recombination must occur not only in a certain pattern but also completely within a region of 85 bp. Nonhomologous recombination events extending beyond the *supF* gene locus would destroy critical vector genes, and the resulting molecules would not be recovered in the assay. Therefore, our analysis may significantly underestimate the ability of triplex-targeted damage to stimulate recombination. This constraint on recombination events in a *supF*-based shuttle vector assay was also evident in a study reporting a low level of interplasmid recombination in experiments in which psoralen adduct formation was carried out exclusively in vitro, where a high level of photomodification would be expected (39). Hence, considering the requirements of the *supF* assay, it is promising that we could still induce detectable recombination and gene conversion via intracellular triplex formation.

The work presented here demonstrates the possibility of using modified oligonucleotides to enhance site-specific recombination and gene conversion within mammalian cells. We have shown that TFOs can serve as sequence-specific DNA binding ligands to deliver damage to a selected gene inside cells. The targeted damage can provoke DNA metabolism, creating recombinogenic substrates. Such oligonucleotides may eventually constitute attractive candidates for use in novel strategies to enhance homologous recombination in gene therapy. Psoralens are probably not the optimal reagents to initiate recombination because they also induce a relatively high level of mutations at the target site, but they are useful model compounds that can be replaced by more efficient DNA cleavage reagents as they are developed. The assay system used here featured duplicated reporter gene sequences in an extrachromosomal vector. The capacity of triplex reagents to modify a chromosomal site and their ability to stimulate recombination between an exogenous DNA molecule and a chromosomal locus, as might be required in a gene therapy application, remain to be determined.

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