[Frontiers in Bioscience 12, 4288-4297, May 1, 2007]

Repair and recombination induced by triple helix DNA

Joanna Y. Chin[1], Erica B. Schleifman[1], Peter M. Glazer[1]

[1]*Departments of Genetics and Therapeutic Radiology, Yale University School of Medicine, New Haven CT*

TABLE OF CONTENTS

1. ABSTRACT

Triple-helix DNA structures can form endogenously at mirror repeat polypurine/polypyrimidine sequences or by introduction of triplex-forming oligonucleotides (TFOs). Recent evidence suggests that triple helices are sources of genetic instability, and are subject to increased rates of mutagenesis and recruitment of repair factors. Indeed, observations using TFOs suggest that triple helices provoke a variety of biological processes which can be harnessed to modulate gene expression and induce heritable changes in targeted genes. This review surveys the biological applications of TFOs, with particular attention to their recombinogenic and mutagenic potential, and summarizes available evidence for the mechanism of triplex and triplex-associated repair.

2. INTRODUCTION

In the half-century since the first triple helical structures were reported (1), significant progress has been made in the application of triplex-forming oligonucleotides (TFOs) as tools for targeted modification of the mammalian genome. The ability of TFOs to bind to duplex DNA tightly, and in a sequence-specific manner, are the key features that underscore the potential of these synthetic oligonucleotides to direct gene manipulation. In the past 20 years, TFOs have been successfully used to inhibit transcription initiation and elongation, induce site-specific damage and cleavage, and target disease-related genes for site-directed mutagenesis or correction. This review will focus on the use of triplex-forming oligonucleotides for targeted recombination and DNA repair.

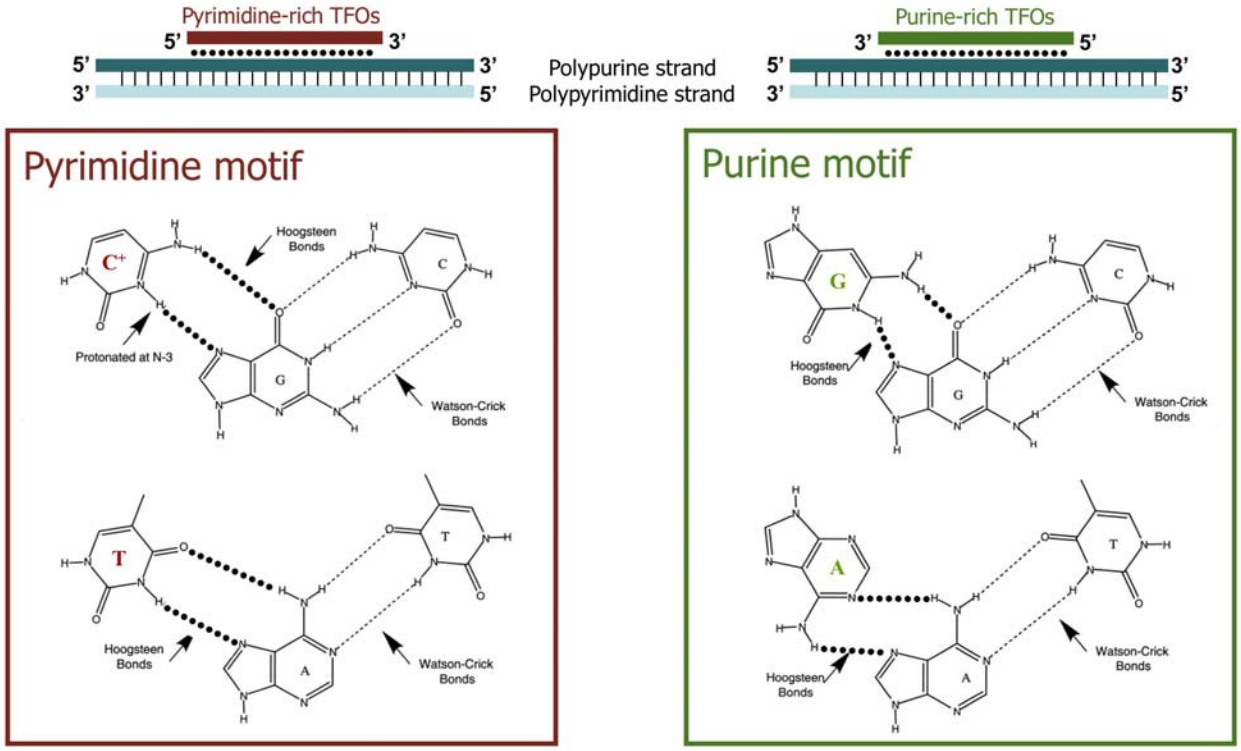

Figure 1. Triplex binding code in the purine-rich and pyrimidine-rich TFO motifs.

TFOs bind in the major groove of duplex DNA via two Hoogsteen hydrogen bonds formed between the base of the third strand and the purine base of the duplex DNA. Third-strand binding occurs more slowly than traditional Watson-Crick basepairing, but triplexes are stable once formed. There is evidence that duplex DNA undergoes helical distortions upon TFO binding (2) and that these distortions trigger endogenous recombination and repair mechanisms in the cell (to be discussed below). The presence of divalent cations Mg^{2+}, Ca^{2+} and Zn^{2+} can augment triplex stability, as can naturally occurring polyamines such as spermine, spermidine, and putrescine (3, 4), by reducing the electrostatic repulsion among the three phosphate backbones.

Triplex formation occurs in two motifs, distinguished by the orientation of the third strand with respect to the polypurine target sequence in the duplex DNA. Generally, pyrimidine-rich TFOs bind in a parallel manner, while purine-rich TFOs bind in an anti-parallel orientation (Figure 1). In the pyrimidine motif, thymines or protonated cytosines of the TFO bind to A:T or G:C Watson-Crick basepairs, respectively, forming the canonical base triads T:AT and C+:GC. Typically, triplex formation with pyrimidine TFOs occur only under acidic conditions, since N3 protonation of the third strand cytosine is required for proper Hoogsteen bonding to the N7 of duplex guanine (5). For this reason, 5' methylcytosine (5'MeC) substitution is widely used to alleviate limitations of natural cytosine, as 5'MeC has a higher pK value than does cytosine. Upon binding, the increased triplex stability and affinity are thought to be due to stacking interactions and the exclusion of water from the major groove (6). Pseudoisocytidine is another cytosine replacement that remains protonated in physiological conditions, and is particularly effective in TFOs containing consecutive cytosines. In fact, a TFO with pseudoisocytidine substitutions has been shown to target the human beta-globin gene in living cells, while the equivalent TFO with 5'MeC substitution did not form stable triplexes at physiological pH (7).

The purine-rich motif binds to DNA in an anti-parallel direction via reverse Hoogsteen bonds to form the base triads G:GC and A:AT. In contrast to the pH requirement of pyrimidine-rich TFOs, effective binding for the purine TFOs requires no protonation, allowing them to bind at neutral pH. However, the guanines in purine-rich oligonucleotides are apt to form G-tetrad structures in physiologic concentrations of K+; such intermolecular complexes presumably reduce the extent of bioavailable TFO (8, 9). To overcome this, 7-deazaxanthine has been used in place of A or T in anti-parallel TFOs. These TFOs can form triplexes with high affinity even in physiological K+ concentrations, and can bind to and induce mutagenesis in an intracellular gene target (10).

TFOs can be composed of DNA, RNA, or synthetic molecules that mimic nucleobases or the phosphoribose backbone. Examples of backbone modifications are the phosphoramidate linkages *N,N*-diethylethylenediamine (DEED) and *N,N*-dimethylaminopropylamine (DMAP), in which a 3' non-bridging oxygen in the sugar is replaced with nitrogen,

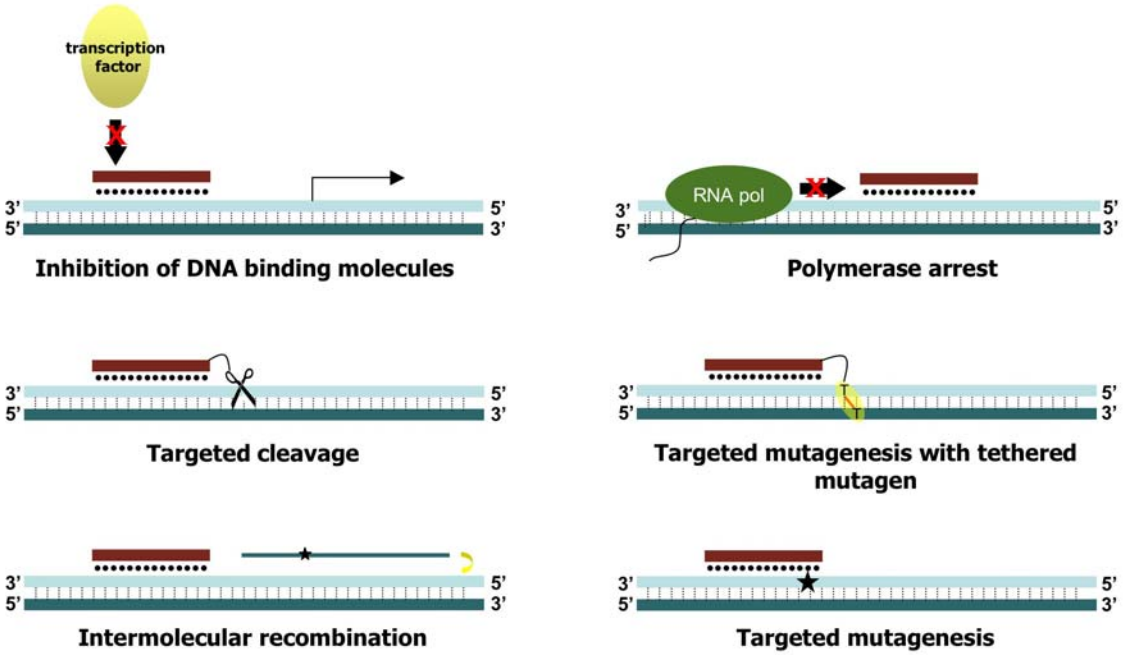

Figure 2. Examples of TFO applications, including modulation of gene expression, targeted delivery of DNA-reactive molecules, and targeted gene modification.

thereby creating a positively charged backbone. TFOs containing these modifications have improved binding affinity and intracellular delivery compared with their phosphodiester counterparts (11-13). Peptide nucleic acids (PNAs), which are synthetic oligonucleotide mimics with a peptide-like backbone, can also form triplex structures with DNA, but their bioactivity and repair are not within the scope of this review (14).

3. BIOLOGICAL APPLICATIONS

An early application of TFOs was site-specific blockage of protein-DNA interactions, such as prevention of endonuclease cleavage (15, 16) or transcription factor binding (17, 18) (Figure 2). Several groups recognized that gene expression could be inhibited when TFOs are bound to promoter elements, presumably by preventing transcription factor binding or by directly inhibiting progression of RNA polymerase. For example, triplex formation at a G-rich site 115 bases upstream from the *c-myc* gene prevented promoter element binding and reduced c-myc expression by ten-fold (19, 20). The binding of these G-rich TFOs at various promoters occurred at physiological pH (21), and nuclear uptake was evident within two hours of transfection into HeLa cells (20). Following this pioneering work, several laboratories have successfully targeted TFOs to a variety of other promoters to arrest target gene transcription, including *Ha-ras*, the *HER2/neu/c-erbB2* proto-oncogene, the alpha subunit of the interleukin-2 receptor, and *cyclin D1* (22-25).

In addition to binding at promoter elements, triplex has been used to prevent transcription elongation by physically blocking the progression of the RNA polymerase complex. Faria and colleagues used a 15-mer mixed-sequence TFO with N3'-P5' phosphoramidate linkages to target a polypurine tract in the coding region of two HIV-1 genes. Using both transient and stable expression systems, the authors detected a 40-50% decrease in reporter RNA expression, but no changes in expression of control genes, implying specific polymerase arrest (26). Work by Krasilnikov and colleagues have shown that polymerase arrest during replication is due to the inability of various DNA polymerases to dissociate triplex structures. That is, significantly higher temperatures were needed to overcome intramolecular triplexes than the corresponding duplex sequences, with the difference in the range of 20-40 degrees (27).

The ability of TFOs to target specific sequences can be used to site-specifically deliver DNA reactive molecules. Various TFO conjugates, for example nucleases, psoralen, radionuclides, and alkylating agents, have been used to induce site-specific DNA damage in yeast and human chromosomes (28-32). One recent example was the delivery of the anthrocycline daunomycin into prostate cancer cells using TFO-drug conjugates to target to *c-myc* promoters (33). DU145 cells express c-myc constitutively, and reduction of c-myc expression is sufficient to cause cancer cell death and tumor regression in mice. After four days of daunomycin-TFO treatment, c-myc expression in these cells was reduced, leading to growth inhibition and a two-fold increase in apoptosis. Of the cells undergoing apoptosis, as assayed by annexin-V staining, more than 80% showed oligonucleotide uptake. In contrast, daunomycin conjugation to an oligonucleotide that lacked triplex-forming ability had no effect on cell growth, and dauno-TFO treatment of normal fibroblasts did

not lead to growth inhibition in doses that killed DU145 cells. Apoptosis and cellular uptake were dose-dependent, providing evidence that cell death was selective for TFO-targeted cells.

Finally, a TFO was used to direct an anti-tumor agent to induce site-specific cleavage in plasmid (34). VP16 (etoposide) prevents topoisomerase II-religation after DNA cleavage, resulting in DNA strand breaks that trigger repair pathways. The VP16-TFOs designed by Duca *et al.* specifically induced double-stranded breaks 13-14 bp from the triplex binding site. Using TFOs to direct cleavage at a particular gene or genomic site can potentially reduce nonspecific DNA damage by VP16. Chemotherapy-associated nonspecific DNA damage is thought to cause translocation events that then lead to secondary cancers, so it is significant that TFOs can decrease nonspecific DNA damage by targeting drug delivery to relevant sites.

3.1. Enhanced recombination

There has been considerable interest in invoking the endogenous homologous recombination machinery to correct or modify chromosomal targets. This strategy, however, has been limited by a low frequency of homologous integration, a high frequency of random integration of vector DNA, and the need for an isolation procedure to select for the corrected population of cells. The frequency of homologous recombination can be augmented by DNA damage, for example by agents that induce double-stranded breaks, which include UV radiation, carcinogens, and the restriction endonuclease I-Sce I (35). As well, photoreactive molecules such as psoralen can create interstrand crosslinks that can efficiently induce recombination in yeast and mammalian cells (36). Because TFOs induce DNA repair in a site-specific manner, they are potentially interesting candidates to enhance recombinogenic frequencies, while also reducing random integration.

To study TFO-induced recombination, our lab designed an SV40 shuttle vector with two tandem copies of the suppressor tRNA reporter gene *supF*, each with a single, unique and inactivating point mutation, separated by a polypurine triplex-binding site. TFOs were bound overnight to this vector and then allowed time to repair either in cell extracts or mammalian cells. The vector was then recovered and used to transform *lacZ* (amber) *E. coli* strains for genetic analysis of the *supF* gene. With this tandem *supF* vector assay, targeted damage at the triplex-binding site was found to stimulate intramolecular recombination, producing one wild-type, functional *supF* gene. Our lab showed that G-rich TFOs could stimulate recombination at a frequency of 0.37% to generate a functional *supF* gene, five-fold above background. Furthermore, TFO conjugation to psoralen augmented the frequency to 1.5%, a 25-fold increase above background (37). Sequencing of revertants suggested a mechanism consistent with nonconservative recombination and gene conversion. As well, extrachromosomal recombination could be induced by a psoralen-conjugated TFO at a frequency 65-fold above background (37). Other groups have similarly shown that TFOs, particularly when conjugated to psoralen, can sensitize genomic sites for modification, producing low but observable frequencies of homologous recombination (38).

An intermolecular recombination strategy was utilized by Chan *et al.*, using a TFO tethered by a linker to a 40-44 nucleotide "donor" molecule. This donor oligo was designed to be homologous to the targeted gene, in this case an extrachromosomal *supF* reporter gene, with one critical difference: the donor oligo contained a desired sequence change that would restore gene activity. In this scenario, the TFO would stimulate repair in the plasmid, and position the donor oligo to provide a source for homologous recombination. Homologous recombination events will then be detectable by assaying for *supF* activity, which would have been restored by incorporation of the sequence change in the donor oligo. Using this approach, Chan *et al.* were able to obtain gene reversion at a frequency of 0.7%, four-fold greater than using a donor oligo alone or with a non-specific TFO, and more than 200-fold above frequencies of spontaneous reversion (39).

The recombinogenic potential of TFOs has also been studied in a single-copy chromosomal locus in mammalian cells, in which TFOs are introduced into Chinese hamster ovary (CHO) cells at the same time as a single-stranded 51-mer donor oligonucleotide (40). In this case, recombination of the donor oligo corrects a single basepair mutation that renders the *firefly luciferase* reporter gene completely inactive. TFOs of both purine and pyrimidine motifs were able to provoke gene correction, restoring luciferase activity at frequencies of 0.11%, seven-fold above that induced by donor oligo alone. In contrast, scrambling the sequence of either the TFO, or the TFO binding site, caused no difference in recombination events relative to background, indicating that the effect is specific to TFOs and that treatment with unrelated oligo by itself does not invoke recombinatory mechanisms. Although this study used a recombination target site that was 70 basepairs away from the TFO binding site, recombination has been observed even with target distances of several hundred basepairs (41).

3.2. Targeted mutagenesis

Related to its use to augment recombination for gene correction, triplex formation can be used to target mutations to specific sites in the genome, thereby inducing heritable changes in gene function and expression. Targeted mutagenesis has indeed been a successful application of TFOs, as shown both *in vitro* and *in vivo*. Psoralen-conjugated, purine-rich TFOs targeting a modified *supF* reporter gene within SV40 shuttle vector DNA produced mutagenesis frequencies in the range of 0.23% to 2.1%, depending on the length of the TFO, the vector, and other conditions. These frequencies represent a 70-100 fold increase of mutagenesis frequency over background (42, 43). Further work demonstrated that neither psoralen conjugation nor photoactivation were an absolute requirement for TFO-mediated mutagenesis in mammalian cells; that is, while the presence of directed psoralen crosslinks can augment mutagenic potential, triple helices by themselves are mutagenic. TFO-induced rates of

mutagenesis exceed those produced by nonspecific duplex crosslinking by free 4'-(hydroxyethoxymethyl)-4,5',8-trimethyl-psoralen (HMT) or by a psoralen-conjugated TFO that does not bind the chromosomal target site (44, 45).

TFOs have also been shown to mediate mutagenesis in a chromosomal target, using a psoralen-conjugated pyrimidine-rich TFO that binds to a 17-mer or 23-mer sequence in the Chinese hamster hypoxanthine phosphoribosyl transferase *(hprt)* gene. The TFO-induced mutagenesis frequency was shown to be several hundred-fold above spontaneous mutagenesis (46). The vast majority of induced mutations (85%) were in the triplex binding region; 77% of these had deletions of 4 to 50 bases, and 4% had base substitution mutations at or adjacent to the site of the crosslink. Psoralen-TFOs that showed little binding affinity for the *hprt* target region were not mutagenic above background, and in this case, TFO treatment without photoactivation of the psoralen moiety did not invoke mutagenesis. Whereas in other systems TFOs by themselves are mutagenic, it is evident that for some targets and for some TFO compositions, psoralen-induced interstrand crosslinking is necessary to potentiate mutagenesis above the levels of detection of the assay. Nevertheless, this study provided important evidence that TFOs can bind and induce mutagenesis in chromosomal loci and can accomplish this with high target specificity.

Finally, TFOs can mediate processes of mutagenesis when delivered *in vivo* (47). Transgenic mice, containing a chromosomally integrated *λsupFG1* vector, were *i.p.* injected with 50 mg/kg of TFO for five consecutive days and sacrificed ten days later to determine oligonucleotide uptake. This study utilized AG30, a 30-mer G-rich TFO that was not tethered to any mutagen. Tissue from liver, skin, kidney, colon, small intestine and lung showed five-fold increases in mutation frequency, when compared with tissue from mice treated with control TFO. Importantly, this latter group did not show differences in mutagenesis from PBS-treated mice, indicating that unrelated TFOs are neither mutagenic nor toxic *in vivo*, and can be tolerated with apparently no off-target effects. Further dosing studies are needed to ensure safety and to augment efficiency, but this study shows that TFOs can effectively target chromosomal DNA when delivered *in vivo*.

4. TFO-INDUCED REPAIR

The initial evidence that triplex binding can invoke repair came from DNA repair synthesis assays, in which we observed that TFO-treated *pSupFG1* vectors incorporate alpha-^{32}P-dCTP from supplemented HeLa cell extracts. By contrast, treatment with a control TFO that did not bind the vector did not lead to incorporation of labeled nucleotide, nor did treatment of plasmids that lacked the TFO binding site. Only TFOs that induced high rates of mutagenesis were able to induce DNA synthesis, suggesting that TFO-induced mutagenesis was related to repair. Further, because these TFOs can also inhibit *in vitro* transcription, a mechanism involving transcription stalling by a bound TFO was proposed, which in turn could trigger gratuitous and possibly error-prone repair and mutagenesis (44).

These initial studies also provided some evidence that nucleotide excision repair (NER) factors are required for TFO activity. For example, consistent with other data, a 30-mer purine-rich TFO can induce mutagenesis in mammalian cells at frequencies of 0.27%, 13-fold above spontaneous mutagenesis. In contrast, the frequencies of mutagenesis using the same TFO in two independent cell lines deficient in *XPA* (*Xeroderma pigmentosa* complementation group A, an NER damage recognition factor), or a cell line deficient in Cockayne's syndrome group B (*CSB*), were an order of magnitude lower, on par with spontaneous mutagenesis. *XPA* complementation by cDNA expression restores TFO-induced mutagenesis to wildtype levels, while *CSB* complementation only partially restores mutagenicity. These results indicate that TFO-mediated mutagenesis is related to DNA repair and specifically implicates NER and transcription-coupled repair (44).

Similarly, recombination induced by TFOs is also dependent on the NER pathway. The frequency of gene reversion mediated by TFOs tethered to donor was diminished in cells deficient in *XPA*, and the observed gene frequencies in these deficient cells were similar to that of untreated, wildtype cells. With correction of the *XPA* defect, frequencies of reversion approached that of non-deficient cells at 0.48% (39). It was further shown in an intramolecular recombination assay that repair of both triplex lesions and psoralen-triplex compound lesions was dependent on *XPA* and the endonucleases *XPF* and *XPG*. However, repair was not found to be dependent on the mismatch repair (MMR) recognition and bridging factors *MSH2* nor *MLH1*, respectively (37).

Subsequent studies showed that TFO-induced recombination required *hsRad51*, the human *recA* homolog that mediates strand exchange reactions. Overexpression of *hsRad51* increased recombinatory activity of a TFO tethered to a donor in an episomal system (48). The involvement of recombinogenic factors is not unexpected, as the donor oligonucleotides in our assays are designed to be substrates for homologous recombination. These results are also consistent with the observation that chromosomal gene targeting by TFOs and induced mutagenesis are significantly augmented by treatment during S phase, compared with treatment of quiescent cells (49). This finding is thought to be due to the presence of replication machinery, recombinogenic factors, and a template for repair during S phase. It is also possible that the increased gene targeting is a reflection of increased gene accessibility and local chromatin remodeling that takes place during S phase.

The extent of triplex repair is related to the recombinogenic potential of the TFO. A recent study tested a variety of chemically modified pyrimidine-rich TFOs for recombinogenic potential in plasmids and CHO cells with a chromosomally integrated TFO binding site. A

TFO containing a phosphoramidate backbone and a TFO containing cytosine and thymine base substitutions both provoked significant levels of recombination (0.3%-0.4%) over background. In contrast to other tested TFOs that had limited *in vitro* triplex binding ability and no bioactivity, plasmid treatment by these same TFOs led to significant incorporation of labeled nucleotide in the DNA repair synthesis assay. Of note, the TFOs that contained 2'OH (RNA) and 2'-O-methyl (2'-O-Me) ribose moieties, which are predicted to provoke the least helical distortion upon third strand binding, provoked insignificant levels of recombination and repair over background (50). These results are consistent with the hypothesis that one of the recognition events of a triplex lesion may be the altered helical structure, an idea that is supported by data on the repair of endogenous triplex structures, explored below.

4.1. Endogenous triple helices as sources of genetic instability

The idea that triple-helix formation provokes genome modification is supported not only by the studies mentioned above, but also by studies of endogenous triple helices found in the human genome. H-DNA, or intramolecular triplexes, occur at purine-pyrimidine tracts with mirror repeat symmetry. It is now apparent that H-DNA-forming sequences occur abundantly within the genome, primarily in promoter regions (51).

H-DNA structures can lead to local genomic instability and breakpoints, resulting in chromosomal translocations. For example, multiple sites of mirror repeats in an unusually long (2.5kb) poly(R-Y) tract in the polycystic kidney disease 1 (*PKD1*) gene have been shown to adopt non-B conformations, including intramolecular triplexes. When this tract is introduced into plasmids and subsequently transduced into *E. coli*, a dramatic loss of viability of colony-forming units results (52). Furthermore, cells lacking *UvrA* or *UvrB* of the nucleotide excision pathway did not exhibit this loss of viability, indicating that intact NER machinery is also required to recognize the altered structures in *E. coli*. The presence of the poly(R-Y) tract also induced an increased frequency of rearrangement mutagenesis in a reporter gene, and sequence analysis in mutated bacteria indicated the presence of breakpoint junctions in areas predicted to form non-B-DNA structures (53). A transcription-related process may be involved in induced mutagenesis, since transcription increased the frequency of *PKD1*-tract induced plasmid alterations. As non-B-DNA conformations are expected to increase the frequency of double-stranded breaks, it is suggestive that these breaks are the initiating events that lead to mutagenesis.

The genetic instability resulting from H-DNA structures has also been observed in mammalian cells, using H-DNA-forming sequences endogenous to the human *c-myc* promoter. Disease-causing translocations of chromosome 8, such as Burkitt's lymphoma, juxtapose the *c-myc* gene next to immunoglobulin enhancer elements, leading to c-myc overexpression. Notably, breakpoints have been found in the area of the H-DNA-forming *c-myc* promoter, suggesting that genetic instability at this site and the resulting disease are direct consequences of altered DNA conformations. To test this, the 23bp, H-DNA-forming region from the human *c-myc* promoter was placed upstream of the *supF* reporter gene. The presence of this H-DNA-forming sequence induced a 20-fold higher frequency of mutagenesis in *supF* than did a scrambled sequence. Notably, mutagenesis induction was coincident with the ability to form H-DNA structures. These induced mutations were revealed to be primarily deletions, but also included insertions, inversions and duplications. Breakpoints demonstrated areas of microhomologies, and a higher frequency of double-stranded breaks were found close to the H-DNA-forming locus, suggesting repair consistent with non-homologous end-joining (54). These results, with similar observations in the t(14;18) oncogenic translocation (55, 56), suggest that non-B-DNA structures are involved in genetic instability, leading to chromosomal rearrangements and disease.

4.2. Repair of complex psoralen-TFO lesions

Repair of triplex structures has also been studied in the context of TFO-directed psoralen-crosslinks. As reviewed above, the presence of the third strand by itself induces repair, but this repair is difficult to detect in mammalian cells, given the barriers to TFO intracellular delivery and triplex formation. Once formed, the triplex structure may be unwound by DNA helicases (57), and interactions with repair machinery are likely to be transient in nature, further hampering detection. In contrast, the covalent lesion caused by psoralen crosslinking readily allows for detection, and thus facilitates identification and study of repair factors that are recruited to these sites. However, as psoralen-induced monoadducts and interstrand crosslinks can provoke repair by themselves, the addition of the third strand complicates the repair process. In reviewing the literature, we should keep in mind that repair of triplex structures may differ from repair of complex lesions caused by psoralen-TFO, and a more rigorous study of their related repair processes is necessary for comparison.

As with triplex structures, there is evidence that TFO-directed psoralen interstrand crosslinks are recognized by NER factors. Replication protein A (RPA), a highly conserved recognition factor, binds specifically to psoralen-monoadducted or psoralen-crosslinked triplex DNA with high affinity. In contrast, RPA does not bind to duplex DNA by itself, whether or not the DNA is crosslinked. XPA acts in a complex with RPA to reduce nonspecific DNA aggregation, although interestingly, XPA does not bind to triplex DNA in the absence of RPA (58). Further studies also implicate a complex of Xeroderma pigmentosa protein Group C (XPC) and the human homolog of RAD23B (hHR23B) in rapid (<10 sec) recognition of triplex-directed psoralen interstrand crosslinks formed *in vitro* (59).

Another NER factor, the endonuclease XPF, has also been studied by several groups. Degols *et al.* demonstrated that XPF was necessary for repair of TFO-directed psoralen crosslinks in plasmid, using inhibition of reporter gene expression as a measure of crosslink presence

and thus, lack of repair (60). Another group, using a psoralen-conjugated TFO that targeted the *hprt* gene, observed an increase in mutation frequency in cells that lacked *XPF* or *ERCC1* (which acts in concert with XPF) relative to wildtype cells. In addition, they observed that lack of *XPF* or *ERCC1* increased the percentage of deletion mutations, while decreasing the frequency of base substitutions, suggesting that XPF/ERCC1 is involved in translesion repair synthesis that leads to base substitutions (61). Sequencing of the TFO-induced deletion mutants provided evidence for a non-homologous end-joining (NHEJ) mechanism, consistent with observations by others (37, 43). That the character of mutations may change (*e.g.* deletions versus base substitutions) suggests there may be multiple, perhaps competing, repair pathways that metabolize this complex lesion.

In fact, in the presence of donor sequence for crosslink repair, NER can suppress intramolecular recombination. This was observed in a plasmid-based crosslink repair assay by Zheng *et al.* in which tandem reporter genes flank a TFO-binding and crosslinking site. NER-deficient cells (lacking XPA or XPF) had a recombination frequency of 0.55%, compared with 0.18% in proficient cells. Because sequencing of the site revealed only one functional copy of the reporter gene, the authors hypothesized that the presence of the TFO-linked crosslink induced single-strand annealing that was suppressed by NER (62).

Mismatch repair is notable for its lack of involvement in TFO-induced mutagenesis. Several groups have now shown that cells that are deficient in the *MutS* and *MutL* homologs *MSH2*, *MLH1*, *MSH3*, or *MSH6*, do not show any change in TFO-induced mutagenesis frequency or mutation type relative to wildtype cells (37, 61, 63). In contrast, Zhang *et al.* demonstrated a requirement for hMutSbeta (*i.e.,* the heterodimer MSH2-MSH3) in recognition and uncoupling of a TFO-associated crosslink in plasmid (64). This apparent conflict led one group to suggest that MMR is involved in error-free processing of TFO-directed psoralen crosslinks (63). In support of this, Zheng *et al.* showed that cells deficient in *MSH2* exhibited an increase in crosslink repair in a gene reactivation assay that required recombination-independent repair. Therefore, MMR heteroduplex resolution may be associated with error-free, recombinational repair (62).

Finally, several studies suggest that the presence of a third strand may impair processing of a TFO-directed psoralen interstrand crosslink. Plasmids containing a triplex binding site from the *IL-2Ralpha* promoter region were treated with pso-TFO or pso-S-S-TFO and transfected into HeLa cells for repair. Guieysse *et al.* observed that the presence of the third strand led to persistent gene inhibition of a reporter gene by the crosslink and to a lack of excision products. In contrast, plasmids in which the third strand was removed (via reduction of the disulfide linker in pso-S-S-TFO) efficiently recovered gene expression and demonstrated excision of the crosslink fragment (65). In an *in vitro* assay measuring labeled nucleotide incorporation in a plasmid, the presence of a bound TFO precluded incision, repair synthesis and religation of the interstrand crosslink; in contrast, these events were observed in a plasmid containing a psoralen crosslink in the absence of a TFO (66).

Inhibition of repair by the third strand may be correlated with TFO length. A 10 nucleotide (10nt) TFO and a 30nt TFO were compared with regard to mutagenic potential and induction of incision. The 30nt TFO provoked a reduced number of detected incisions than DNA treated with the 10nt TFO, suggesting that the longer TFO may inhibit access to a 5' incision site, thus reducing repair efficiency (67). Since the 30nt TFO had a higher binding affinity for the target duplex site than did the 10nt TFO, the 30nt TFO may also be a better competitor with potential NER proteins. The authors observed that the 10nt TFO generated mostly A:T to T:A transversions in a reporter gene, while the 30nt TFO generated a diverse set of mutations, including large deletions. As above, the difference in the character of the mutants may indicate alternate repair pathways, in this case dependent on the length of the TFO and possibly the TFO binding affinity. These results indicated that the presence of the third strand influences repair of the psoralen-adduct and points to the complexity in studying triplex-associated lesions.

5. PERSPECTIVE

TFOs have enormous potential as agents for gene modification and modulation, but some key issues remain to be resolved prior to clinical application. It is clear from the studies reviewed here that TFOs are inherently mutagenic and recombinogenic when bound to their intended target sites, although the mechanisms of these events are still under investigation. Further studies of how triplex structures are recognized and repaired will inform our ongoing efforts to improve efficiency and targeting of disease-causing genes.

6. ACKNOWLEDGEMENTS

We gratefully acknowledge members of the Glazer Lab for helpful discussions. This work was supported by a grant from the NIH (R01CA64186) to P.M.G., a grant from the NIH MSTP (Training Grant 5T32GM07205) to J.Y.C., and a grant from the NIH Genetics Training Grant to E.B.S..

7. REFERENCES

1. Felsenfeld, G., D. R. Davies & A. Rich: Formation of a three-stranded polynucleotide molecule. *J Am Chem Soc*, 79, 2023-2024 (1957)
2. Hartman, D. A., S. R. Kuo, T. R. Broker, L. T. Chow & R. D. Wells: Intermolecular triplex formation distorts the DNA duplex in the regulatory region of human papillomavirus type-11. *J Biol Chem*, 267, 5488-94 (1992)
3. Hampel, K. J., P. Crosson & J. S. Lee: Polyamines favor DNA triplex formation at neutral pH. *Biochemistry*, 30, 4455-9 (1991)
4. Thomas, T. & T. J. Thomas: Selectivity of polyamines in triplex DNA stabilization. *Biochemistry*, 32, 14068-74 (1993)

5. Povsic, T. J. & P. B. Dervan: Triple helix formation by oligonucleotides on DNA extened to the physiological range. *J. Am. Chem. Soc*, 111, 3059-3061 (1989)
6. Xodo, L. E., G. Manzini, F. Quadrifoglio, G. A. van der Marel & J. H. van Boom: Effect of 5-methylcytosine on the stability of triple-stranded DNA-a thermodynamic study. *Nucleic Acids Res*, 19, 5625-31 (1991)
7. Shahid, K. A., A. Majumdar, R. Alam, S. T. Liu, J. Y. Kuan, X. Sui, B. Cuenoud, P. M. Glazer, P. S. Miller & M. M. Seidman: Targeted cross-linking of the human beta-globin gene in living cells mediated by a triple helix forming oligonucleotide. *Biochemistry*, 45, 1970-8 (2006)
8. Sen, D. & W. Gilbert: A sodium-potassium switch in the formation of four-stranded G4-DNA. *Nature*, 344, 410-414 (1990)
9. Noonberg, S. B., J. C. Francois, T. Garestier & C. Helene: Effect of competing self-structure on triplex formation with purine-rich oligodeoxynucleotides containing GA repeats. *Nucleic Acids Res*, 23, 1956-63 (1995)
10. Faruqi, A. F., S. H. Krawczyk, M. D. Matteucci & P. M. Glazer: Potassium-resistant triple helix formation and improved intracellular gene targeting by oligodeoxyribonucleotides containing 7-deazaxanthine. *Nucleic Acids Res*, 25, 633-40 (1997)
11. Dagle, J. M. & D. L. Weeks: Positively charged oligonucleotides overcome potassium-mediated inhibition of triplex DNA formation. *Nucleic Acids Res*, 24, 2143-9 (1996)
12. Vasquez, K. M., J. M. Dagle, D. L. Weeks & P. M. Glazer: Chromosome targeting at short polypurine sites by cationic triplex-forming oligonucleotides. *J Biol Chem*, 276, 38536-41 (2001)
13. Michel, T., F. Debart, F. Heitz & J. J. Vasseur: Highly stable DNA triplexes formed with cationic phosphoramidate pyrimidine alpha-oligonucleotides. *Chembiochem*, 6, 1254-62 (2005)
14. Nielsen, P. E.: PNA Technology. *Mol Biotechnol*, 26, 233-48 (2004)
15. Francois, J. C., T. Saison-Behmoaras, N. T. Thuong & C. Helene: Inhibition of restriction endonuclease cleavage via triple helix formation by homopyrimidine oligonucleotides. *Biochemistry*, 28, 9617-9 (1989)
16. Hanvey, J. C., M. Shimizu & R. D. Wells: Site-specific inhibition of EcoRI restriction/modification enzymes by a DNA triple helix. *Nucleic Acids Res*, 18, 157-61 (1990)
17. Maher, L. J., B. Wold & P. B. Dervan: Inhibition of DNA binding proteins by oligonucleotide-directed triple helix formation. *Science*, 245, 725-30 (1989)
18. Mayfield, C., S. Ebbinghaus, J. Gee, D. Jones, B. Rodu, M. Squibb & D. Miller: Triplex formation by the human Ha-ras promoter inhibits Sp1 binding and *in vitro* transcription. *J Biol Chem*, 269, 18232-8 (1994)
19. Cooney, M., G. Czernuszewicz, E. H. Postel, S. J. Flint & M. E. Hogan: Site-specific oligonucleotide binding represses transcription of the human c-myc gene *in vitro*. *Science*, 241, 456-9 (1988)
20. Postel, E. H., S. J. Flint, D. J. Kessler & M. E. Hogan: Evidence that a triplex-forming oligodeoxyribonucleotide binds to the c-myc promoter in HeLa cells, thereby reducing c-myc mRNA levels. *Proc Natl Acad Sci U S A*, 88, 8227-31 (1991)
21. Durland, R. H., D. J. Kessler, S. Gunnell, M. Duvic, B. M. Pettitt & M. E. Hogan: Binding of triple helix forming oligonucleotides to sites in gene promoters. *Biochemistry*, 30, 9246-55 (1991)
22. Mayfield, C., M. Squibb & D. Miller: Inhibition of nuclear protein binding to the human Ki-ras promoter by triplex-forming oligonucleotides. *Biochemistry*, 33, 3358-63 (1994)
23. Noonberg, S. B., G. K. Scott, C. A. Hunt, M. E. Hogan & C. C. Benz: Inhibition of transcription factor binding to the HER2 promoter by triplex-forming oligodeoxyribonucleotides. *Gene*, 149, 123-6 (1994)
24. Grigoriev, M., D. Praseuth, A. L. Guieysse, P. Robin, N. T. Thuong, C. Helene & A. Harel-Bellan: Inhibition of gene expression by triple helix-directed DNA cross-linking at specific sites. *Proc Natl Acad Sci U S A*, 90, 3501-5 (1993)
25. Kim, H. G. & D. M. Miller: A novel triplex-forming oligonucleotide targeted to human cyclin D1 (bcl-1, proto-oncogene) promoter inhibits transcription in HeLa cells. *Biochemistry*, 37, 2666-72 (1998)
26. Faria, M., C. D. Wood, L. Perrouault, J. S. Nelson, A. Winter, M. R. White, C. Helene & C. Giovannangeli: Targeted inhibition of transcription elongation in cells mediated by triplex-forming oligonucleotides. *Proc Natl Acad Sci U S A*, 97, 3862-7 (2000)
27. Krasilnikov, A. S., I. G. Panyutin, G. M. Samadashwily, R. Cox, Y. S. Lazurkin & S. M. Mirkin: Mechanisms of triplex-caused polymerization arrest. *Nucleic Acids Res*, 25, 1339-46 (1997)
28. Le Doan, T., L. Perrouault, D. Praseuth, N. Habhoub, J. L. Decout, N. T. Thuong, J. Lhomme & C. Helene: Sequence-specific recognition, photocrosslinking and cleavage of the DNA double helix by an oligo-[alpha]-thymidylate covalently linked to an azidoproflavine derivative. *Nucleic Acids Res*, 15, 7749-60 (1987)
29. Moser, H. E. & P. B. Dervan: Sequence-specific cleavage of double helical DNA by triple helix formation. *Science*, 238, 645-50 (1987)
30. Strobel, S. A. & P. B. Dervan: Site-specific cleavage of a yeast chromosome by oligonucleotide-directed triple-helix formation. *Science*, 249, 73-5 (1990)
31. Strobel, S. A., L. A. Doucette-Stamm, L. Riba, D. E. Housman & P. B. Dervan: Site-specific cleavage of human chromosome 4 mediated by triple-helix formation. *Science*, 254, 1639-42 (1991)
32. Arimondo, P. B., A. Boutorine, B. Baldeyrou, C. Bailly, M. Kuwahara, S. M. Hecht, J. S. Sun, T. Garestier & C. Helene: Design and optimization of camptothecin conjugates of triple helix-forming oligonucleotides for sequence-specific DNA cleavage by topoisomerase I. *J Biol Chem*, 277, 3132-40 (2002)
33. Napoli, S., U. Negri, F. Arcamone, M. L. Capobianco, G. M. Carbone & C. V. Catapano: Growth inhibition and apoptosis induced by daunomycin-conjugated triplex-forming oligonucleotides targeting the c-myc gene in prostate cancer cells. *Nucleic Acids Res*, 34, 734-44 (2006)
34. Duca, M., D. Guianvarc'h, K. Oussedik, L. Halby, A. Garbesi, D. Dauzonne, C. Monneret, N. Osheroff, C. Giovannangeli & P. B. Arimondo: Molecular basis of the targeting of topoisomerase II-mediated DNA cleavage by VP16 derivatives conjugated to triplex-forming oligonucleotides. *Nucleic Acids Res*, 34, 1900-11 (2006)

35. Rouet, P., F. Smih & M. Jasin: Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proc Natl Acad Sci U S A*, 91, 6064-8 (1994)
36. De Silva, I. U., P. J. McHugh, P. H. Clingen & J. A. Hartley: Defining the roles of nucleotide excision repair and recombination in the repair of DNA interstrand cross-links in mammalian cells. *Mol Cell Biol*, 20, 7980-90 (2000)
37. Faruqi, A. F., H. J. Datta, D. Carroll, M. M. Seidman & P. M. Glazer: Triple-helix formation induces recombination in mammalian cells via a nucleotide excision repair-dependent pathway. *Mol Cell Biol*, 20, 990-1000 (2000)
38. Sandor, Z. & A. Bredberg: Triple helix directed psoralen adducts induce a low frequency of recombination in an SV40 shuttle vector. *Biochim Biophys Acta*, 1263, 235-40 (1995)
39. Chan, P. P., M. Lin, A. F. Faruqi, J. Powell, M. M. Seidman & P. M. Glazer: Targeted correction of an episomal gene in mammalian cells by a short DNA fragment tethered to a triplex-forming oligonucleotide. *J Biol Chem*, 274, 11541-8 (1999)
40. Knauert, M. P., J. M. Kalish, D. C. Hegan & P. M. Glazer: Triplex-stimulated intermolecular recombination at a single-copy genomic target. *Mol Ther*, 14, 392-400 (2006)
41. Knauert, M. P., J. A. Lloyd, F. A. Rogers, H. J. Datta, M. L. Bennett, D. L. Weeks & P. M. Glazer: Distance and affinity dependence of triplex-induced recombination. *Biochemistry*, 44, 3856-64 (2005)
42. Havre, P. A., E. J. Gunther, F. P. Gasparro & P. M. Glazer: Targeted mutagenesis of DNA using triple helix-forming oligonucleotides linked to psoralen. *Proc Natl Acad Sci U S A*, 90, 7879-83 (1993)
43. Wang, G., D. D. Levy, M. M. Seidman & P. M. Glazer: Targeted mutagenesis in mammalian cells mediated by intracellular triple helix formation. *Mol Cell Biol*, 15, 1759-68 (1995)
44. Wang, G., M. M. Seidman & P. M. Glazer: Mutagenesis in mammalian cells induced by triple helix formation and transcription-coupled repair. *Science*, 271, 802-5 (1996)
45. Vasquez, K. M., G. Wang, P. A. Havre & P. M. Glazer: Chromosomal mutations induced by triplex-forming oligonucleotides in mammalian cells. *Nucleic Acids Res*, 27, 1176-81 (1999)
46. Majumdar, A., A. Khorlin, N. Dyatkina, F. L. Lin, J. Powell, J. Liu, Z. Fei, Y. Khripine, K. A. Watanabe, J. George, P. M. Glazer & M. M. Seidman: Targeted gene knockout mediated by triple helix forming oligonucleotides. *Nat Genet*, 20, 212-4 (1998)
47. Vasquez, K. M., L. Narayanan & P. M. Glazer: Specific mutations induced by triplex-forming oligonucleotides in mice. *Science*, 290, 530-3 (2000)
48. Datta, H. J., P. P. Chan, K. M. Vasquez, R. C. Gupta & P. M. Glazer: Triplex-induced recombination in human cell-free extracts. Dependence on XPA and HsRad51. *J Biol Chem*, 276, 18018-23 (2001)
49. Majumdar, A., N. Puri, B. Cuenoud, F. Natt, P. Martin, A. Khorlin, N. Dyatkina, A. J. George, P. S. Miller & M. M. Seidman: Cell cycle modulation of gene targeting by a triple helix-forming oligonucleotide. *J Biol Chem*, 278, 11072-7 (2003)
50. Kalish, J. M., M. M. Seidman, D. L. Weeks & P. M. Glazer: Triplex-induced recombination and repair in the pyrimidine motif. *Nucleic Acids Res*, 33, 3492-502 (2005)
51. Wang, G. & K. M. Vasquez: Non-B DNA structure-induced genetic instability. *Mutat Res*, 598, 103-19 (2006)
52. Bacolla, A., A. Jaworski, T. D. Connors & R. D. Wells: Pkd1 unusual DNA conformations are recognized by nucleotide excision repair. *J Biol Chem*, 276, 18597-604 (2001)
53. Bacolla, A., A. Jaworski, J. E. Larson, J. P. Jakupciak, N. Chuzhanova, S. S. Abeysinghe, C. D. O'Connell, D. N. Cooper & R. D. Wells: Breakpoints of gross deletions coincide with non-B DNA conformations. *Proc Natl Acad Sci U S A*, 101, 14162-7 (2004)
54. Wang, G. & K. M. Vasquez: Naturally occurring H-DNA-forming sequences are mutagenic in mammalian cells. *Proc Natl Acad Sci U S A*, 101, 13448-53 (2004)
55. Raghavan, S. C., P. C. Swanson, X. Wu, C. L. Hsieh & M. R. Lieber: A non-B-DNA structure at the Bcl-2 major breakpoint region is cleaved by the RAG complex. *Nature*, 428, 88-93 (2004)
56. Raghavan, S. C., P. Chastain, J. S. Lee, B. G. Hegde, S. Houston, R. Langen, C. L. Hsieh, I. S. Haworth & M. R. Lieber: Evidence for a triplex DNA conformation at the bcl-2 major breakpoint region of the t (14;18) translocation. *J Biol Chem*, 280, 22749-60 (2005)
57. Maine, I. P. & T. Kodadek: Efficient unwinding of triplex DNA by a DNA helicase. *Biochem Biophys Res Commun*, 204, 1119-24 (1994)
58. Vasquez, K. M., J. Christensen, L. Li, R. A. Finch & P. M. Glazer: Human XPA and RPA DNA repair proteins participate in specific recognition of triplex-induced helical distortions. *Proc Natl Acad Sci U S A*, 99, 5848-53 (2002)
59. Thoma, B. S., M. Wakasugi, J. Christensen, M. C. Reddy & K. M. Vasquez: Human XPC-hHR23B interacts with XPA-RPA in the recognition of triplex-directed psoralen DNA interstrand crosslinks. *Nucleic Acids Res*, 33, 2993-3001 (2005)
60. Degols, G., J. P. Clarenc, B. Lebleu & J. P. Leonetti: Reversible inhibition of gene expression by a psoralen functionalized triple helix forming oligonucleotide in intact cells. *J Biol Chem*, 269, 16933-7 (1994)
61. Richards, S., S. T. Liu, A. Majumdar, J. L. Liu, R. S. Nairn, M. Bernier, V. Maher & M. M. Seidman: Triplex targeted genomic crosslinks enter separable deletion and base substitution pathways. *Nucleic Acids Res*, 33, 5382-93 (2005)
62. Zheng, H., X. Wang, R. J. Legerski, P. M. Glazer & L. Li: Repair of DNA interstrand cross-links: interactions between homology-dependent and homology-independent pathways. *DNA Repair (Amst)*, 5, 566-74 (2006)
63. Wu, Q., L. A. Christensen, R. J. Legerski & K. M. Vasquez: Mismatch repair participates in error-free processing of DNA interstrand crosslinks in human cells. *EMBO Rep*, 6, 551-7 (2005)
64. Zhang, N., X. Lu, X. Zhang, C. A. Peterson & R. J. Legerski: hMutSbeta is required for the recognition and uncoupling of psoralen interstrand cross-links *in vitro*. *Mol Cell Biol*, 22, 2388-97 (2002)

65. Guieysse, A. L., D. Praseuth, C. Giovannangeli, U. Asseline & C. Helene: Psoralen adducts induced by triplex-forming oligonucleotides are refractory to repair in HeLa cells. *J Mol Biol*, 296, 373-83 (2000)
66. Guillonneau, F., A. L. Guieysse, S. Nocentini, C. Giovannangeli & D. Praseuth: Psoralen interstrand cross-link repair is specifically altered by an adjacent triple-stranded structure. *Nucleic Acids Res*, 32, 1143-53 (2004)
67. Wang, G. & P. M. Glazer: Altered repair of targeted psoralen photoadducts in the context of an oligonucleotide-mediated triple helix. *J Biol Chem*, 270, 22595-601 (1995)

Abbreviations: TFO: triplex-forming oligonucleotide, 5'MeC: 5'methylcytosine, NER: nucleotide excision repair, MMR: mismatch repair

Key words: TFO, Triplex-Forming Oligonucleotides, H-DNA, DNA repair, Nucleotide Excision Repair, Anti-Gene, Mutagenesis, Homologous Recombination, Gene Correction, Review

Send correspondence to: Dr Peter M. Glazer, Department of Therapeutic Radiology, Yale University School of Medicine, 333 Cedar St., PO Box 208040, New Haven, CT 06520-8040, Tel: 203-737-2788, Fax: 203-785-6309, E-mail: peter.glazer@yale.edu

http://www.bioscience.org/current/vol12.htm