Use of Articles in the Pachyonychia Congenita Bibliography

The articles in the PC Bibliography may be restricted by copyright laws. These have been made available to you by PC Project for the exclusive use in teaching, scholarship or research regarding Pachyonychia Congenita.

To the best of our understanding, in supplying this material to you we have followed the guidelines of Sec 107 regarding fair use of copyright materials. That section reads as follows:

Sec. 107. - Limitations on exclusive rights: Fair use. Notwithstanding the provisions of sections 106 and 106A, the fair use of a copyrighted work, including such use by reproduction in copies or phonorecords or by any other means specified by that section, for purposes such as criticism, comment, news reporting, teaching (including multiple copies for classroom use), scholarship, or research, is not an infringement of copyright. In determining whether the use made of a work in any particular case is a fair use the factors to be considered shall include - (1) the purpose and character of the use, including whether such use is of a commercial nature or is for nonprofit educational purposes; (2) the nature of the copyrighted work; (3) the amount and substantiality of the portion used in relation to the copyrighted work as a whole; and (4) the effect of the use upon the potential market for or value of the copyrighted work. The fact that a work is unpublished shall not itself bar a finding of fair use if such finding is made upon consideration of all the above factors.

We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.
Gene Targeting via Triple-Helix Formation

BRIAN P. CASEY AND PETER M. GLAZER

Departments of Therapeutic Radiology and Genetics
Yale University School of Medicine
New Haven, Connecticut 06520

I. General Problems in Oligonucleotide-Based Gene Therapy Strategies ................................................. 164
II. Delivery of Triplex-Forming Molecules ........................................................................................................ 164
III. Minor-Groove Binders ................................................................................................................................ 166
IV. Peptide Nucleic Acids ................................................................................................................................ 166
V. Basic Chemistry of TFOs ............................................................................................................................... 167
VI. Improvements in TFO Chemistry .................................................................................................................. 170
VII. TFOs as Molecular Tools ............................................................................................................................ 172
VIII. The Chromatin Barrier .............................................................................................................................. 174
IX. TFOs as Antigene Agents ............................................................................................................................ 177
X. TFO-Mediated Upregulation of Gene Expression ....................................................................................... 178
XI. TFO-Mediated Mutagenesis ......................................................................................................................... 180
XII. TFO-Mediated Recombination .................................................................................................................... 181
XIII. TFO-Directed Sequence Change ............................................................................................................ 185
XIV. Repair Systems Implicated in TFO-Induced DNA Alterations ................................................................ 185
XV. Conclusion .................................................................................................................................................. 188
References ......................................................................................................................................................... 189

A report on a recent workshop entitled “Gene-Targeted Drugs: Function and Delivery” conveys a justified optimism for the eventual feasibility and therapeutic benefit of gene-targeting strategies (1). Although multiple approaches are being explored, this chapter focuses primarily on the uses of triplex-forming oligonucleotides (TFOs). TFOs are molecules that bind in the major groove of duplex DNA and by so doing can produce triplex structures. They bind to the purine-rich strand of the duplex through Hoogsteen or reverse Hoogsteen hydrogen bonding. They exist in two sequence motifs, either pyrimidine or purine. Improvements in delivery of these TFOs are reducing the quantities required for an effective intracellular concentration. New TFO chemistries are increasing the half-life of these oligos and expanding the range of sequences that can be targeted. Alone or conjugated to active molecules, TFOs have proven to be versatile agents both in vitro and in vivo. Foremost, TFOs have been employed in antigenic strategies

1Correspondence should be addressed to Peter M. Glazer: telephone (203) 737-2788; fax: (203) 737-2630; E-mail: peter.glazer@yale.edu.
as an alternative to antisense technology. Conversely, they are also being investigated as possible upregulators of transcription. TFOs have also been shown to produce mutagenic events, even in the absence of tethered mutagens. TFOs can increase rates of recombination between homologous sequences in close proximity. Directed sequence changes leading to gene correction have been achieved through the use of TFOs. Because it is theorized that these modifications are due to the instigation of DNA repair mechanisms, an important area of TFO research is the study of triple-helix recognition and repair. © 2001 Academic Press.

I. General Problems in Oligonucleotide-Based Gene Therapy Strategies

Despite great hope for specificity of action, any molecular-based therapeutic strategy can have unintended effects. As human antisense trials are already in reality, it is not premature to study possible immune responses to the delivery vehicle, conjugated chemical agents, or to the nucleic acid itself. Even naked DNA, which might be thought to be the least antigenic of the three, can elicit negative reactions in animals. Bacterial DNA or DNA of unusual geometry has been shown to provoke antibody production (2). In addition, a charged oligo may be sequestered, and so rendered inactive, by charged cellular proteins (2). Phosphorothioate molecules, for example, have documented protein-binding tendencies (3). The length of the oligo also poses a problem. Although an increase in oligo length assures stability of the triplex through increases in hydrogen bonding and base stacking, it does not necessarily assure greater specificity. For any given sequence of an oligo, some erroneous targeting always occurs owing to the binding of a subset of that sequence. Because control and active oligos are necessarily different in at least one important variable (sequence, base type, backbone chemistry, or sugar conformation or chemistry), it is difficult to design a control that would have the same potential for side effects (3). If TFOs are to be used in conjunction with agents that can damage DNA, there is the additional concern of promiscuous actions by the appended moiety.

II. Delivery of Triplex-Forming Molecules

As the therapeutic value of TFOs so heavily will depend upon the ability to deliver sufficient quantities to the nucleus of cells, this review cannot neglect a brief discussion of some of the currently available delivery choices.

The attraction of a vectorless delivery system is the significant lessening of the possibility of an immune reaction. Passive uptake of oligonucleotides, albeit reasonably effective, usually occurs through endocytosis. Known inhibitors of endocytosis can greatly reduce the uptake of oligos (4). Other modes of delivery are being sought because the short half-life in cells of phosphodiester molecules can, to a large extent, be attributed to endosomal destruction. Cationic lipid mixtures have been shown to increase the rates of association of phosphorothioate molecules to cells 25-fold and, more specifically, to increase the amount that can successfully reach the nucleus (5). Streptolysin O produces reversible pores in the cell membrane. Using a concentration of 150 units/ml, reversible permeabilization of 75% of a population of leukemia cells has been detected, and uptake of chimeric fluorescent methylphosphonodiester/phosphodiester oligonucleotide molecules has been increased at least 1000-fold over passive uptake levels (6). Effectively removing the membrane barrier, either through the use of streptolysin O or through electroporation methods, may be unsuitable for therapeutic purposes.

The use of carrier molecules has also been attempted. The highly branched, and hence highly valenced, polyethyleneimine molecule is easily protonated and serves as a good cationic carrier through the negatively charged outer cell membrane. The protonation appears to serve a critical buffering function as well, which allows survival of oligos in endosomes (7). Another way the endosomal pathway can be bypassed involves the use of fusion peptides that have hydrophobic domains for crossing the membrane as well as hydrophilic nuclear localization signals for addressing the oligo. Such a construct has succeeded in transporting 90% of a plasmid into the cytoplasm in mammalian cells in an hour without cytotoxic effects. Under temperature conditions not favorable to the endosomal pathway it was still effective (8). Among the distinct advantages of using a viral delivery system are the ability to escape the endosomal system and the ability to target specific cell types at near-perfect efficiency. Immunohistochemistry of nuclear lysates from cells infected by oligo-linked adenovirus constructs revealed high levels of nuclear localization. The concentration of internalized oligo was predicted to have reached 20 μM (9).

The issue of half-life remains. Despite the protection from endosomes, the half-life of even modified oligos is still typically only about half a day. Pharmacological study of the fate of phosphorothioate molecules injected into mice found, on the positive side, widespread distribution of the oligo among all tissues but the brain, and on the negative side, almost 50% degradation and excretion within 2 days. Different tissues revealed different propensities to degrade the oligo (10). A study done in mice demonstrated an 8-h stability of a purine-rich phosphodiester TFO modified with only a 3' propylamine (11). The enhancement of nuclease resistance should secure a greater chance for success of molecular therapies if repeated or prolonged TFO action is beneficial; however, unforeseen detrimental effects of prolonged TFO existence may arise.
III. Minor-Groove Binders

As an alternative to sequence-specific targeting via the major groove, small minor-groove binding molecules have also been exploited to try to circumvent the concentration, sequence, and ion limitations of TFOs. In 1992 it was reported that a 5-bp mixed sequence (5'-TGTCA-3') was successfully targeted by either of two peptide derivatives of netropsin and distamycin a. Binding of dimers of these peptides to the minor groove is thought to occur through hydrogen bonds formed between nitrogen atoms on the peptides and the N3 of purine, O2 of pyrimidines, and N2 of guanine (12). Many potential antitumor drugs are mutagenic agents that work by binding in the minor groove and producing lesions and/or by inhibiting repair processes (13). If this mutagenic property could be better directed toward specific genome locations, treatments could perhaps be more effective.

Binding rules to duplex pairs based upon the specific apposition of pyrrole and imidazole rings have been derived. In accordance with these rules, a six-nucleotide sequence was successfully targeted at subnanomolar concentrations, and with high specificity, a one-nucleotide change reduced binding by at least an order of magnitude (14). The practical relevance of such small molecules for gene manipulation efforts became clear with the demonstrated relief of repression of a cytomegalovirus gene. In vitro pyrrole-imidazole compounds were able to reduce the binding of a repressor and thus increase transcription (15).

IV. Peptide Nucleic Acids

Peptide nucleic acids (PNAs) are another example of a sequence-specific DNA-binding molecule. These hybrid molecules contain the bases seen in nucleotides, and thus have the same capacity for engaging in Watson–Crick or Hoogsteen hydrogen bonding, but are supported on a peptide backbone. Their chemistry and construction can be found in a 1996 review (16). They are useful for gene-targeting efforts because of their nuclease resistance, neutral charge, and slow rates of dissociation. As with TFOs, various chemical improvements have been made to broaden the ability of PNAs to bind targets under less than optimal conditions (17, 18).

One species of PNAs, bis-PNAs, has two domains that form clamps by binding the same patch of DNA. PNA clamps are characterized by strand displacement and by extremely slow off rates. One PNA domain strand invades, often with the help of attached positively charged residues such as lysine, and binds via the standard Watson–Crick hydrogen bonds to a complementary purine sequence. The other linked domain binds via Hoogsteen bonds in the same manner as a pyrimidine-motif TFO. Which domain binds first has been disputed.

One study looking at kinetic constants concluded that the Watson–Crick domain binds first and furnishes the specificity of binding. The overall binding process seemed to be dictated by the kinetics of duplex formation (19). Another study taking advantage of the need for protonation of the third-strand cytosine came to the opposite conclusion. The binding rates and specificity were pH-dependent and thus due to the Hoogsteen binding domain. If the cytosines were substituted with pseudoisocytosine, the specificity rates went up at neutral pH (20). In any case, active transcription through the PNA target region greatly increases the rate of clamp formation (21).

PNAs have been tested for their ability to interfere with normal DNA processes and for their mutagenic character. (For a review of therapeutic applications of PNAs, see Ref. 22.) A PNA concentration as low as 1 μM has been shown to block in vitro transcription elongation (23). As a testament to the stability of the PNA clamp, the activity level of a viral helicase, which is known to disrupt TFO-formed triplexes, has been reduced to half in in vitro assays using only nanomolar amounts of PNAs (24). Ten-mer PNA clamps introduced through streptolysin O exposure into mouse fibroblast cells induced mutations 10 times above background levels when targeted against incorporated supF genes. Most mutations consisted of one base-pair insertion or deletions around the clamp site, suggestive of strand slippage events (25).

V. Basic Chemistry of TFOs

While attempting to solve the structure of DNA, James Watson pondered the idea that life may depend on a triad. The initial data before him, it seemed, equally fit a three-stranded model (26). Just a few years after the publication of Watson and Crick's "suggested" double helix, a new type of DNA assembly was discovered. Work with strands of polyuridine and polyadenine unexpectedly led to complexes possessing a 2 : 1 ratio which were further stabilized in the presence of divalent cations (27). Although it was then established that such triplex structures can be artificially created, the degree to which they actually form in vivo remains uncertain.

In vitro studies indicate that triple-helix formation is slower by several orders of magnitude than that of duplexes (28). Nevertheless, intramolecular triplexes, which do not involve foreign strands, have since been shown to form spontaneously in vitro with sequences taken from the promoter regions of natural genes. As a result, potential roles for triple helices in gene regulation have been posited (29). Further indirect evidence that such structures may form under natural conditions, and hence are readily recognized, comes from an in vitro study indicating that bacterial transposons may use triplex structures as guides in choosing integration sites (30), and from the demonstration that triplex-binding proteins exist in mammalian nuclear cell extracts (31).
The discovery of triple-strand binding naturally led to an investigation of sequence requirements. Using columns with fixed single strands, the binding rules were worked out by observing which duplexes could be retained on the columns under different pH and temperature conditions (32). It was discovered that a potential target duplex must contain a strand with a run of purines, for only a purine-rich strand can provide the necessary hydrogen bonding ability to form triple helices (33). From these experiments, it is known that degeneracy exists in the binding rules: A can bind to A, U, and T, while G can bind G or C (32). Three TFO sequence motifs are possible: one in which the third strand consists of the purines G and A, another in which the pyrimidines C and T compose the oligo, and finally a mixed purine/pyrimidine TFO with G and T as the constituent nucleotides. All varieties bind in the major groove without strand invasion. Evidence for duplex unwinding at the site of DNA triplex formation is lacking (34). However, duplex invasion with a phosphodiester molecule is possible if an invading oligo is tethered to a triplex-forming molecule (35). Experiments carried out using oligos designed to deliver cleaving agents to a duplex/triplex junction yielded patterns indicating that purine and mixed purine/pyrimidine third strands bind antiparallel (in reference to their 3' to 5' phosphate orientations) to the purine target via reverse Hoogsteen hydrogen bonds, whereas pyrimidine strands bind in a parallel manner through Hoogsteen bonds (Fig. 1). Molecular modeling has revealed that in the purine motif, because of the need to keep the
bases in the anti conformation, the ribophosphate backbone is placed approximately equidistant from each duplex strand. The same is not true in the case of a pyrimidine motif (36).

Kinetic and thermodynamic studies have uncovered properties essential for triplex formation. Activation energy calculations have shown that three to five triplets must be bound before subsequent rapid formation of a stable triplex is possible. Centrally located mismatches apparently cause no difference in on rates but do increase the off rates (38). Studies substituting 5-methyl cytosine for C in pyrimidine TFOs have shown the importance of stacking interactions for the stabilization of triple helices (0.1–0.4 kcal/mol over a pH range of 5.8–7.6) (37). (For a discussion of the kinetic properties of pyrimidine triplex formation, see Ref. 38.)

Each TFO motif is plagued by a major limitation. The purine motif and the mixed motif with a high G content require that the concentration of monovalent cations, particularly K⁺, be extremely low, below physiological amounts. This is because such ions can stabilize unwanted TFO secondary structure, like G quartets, thereby lessening the supply of oligos poised for triplex formation. Gel shift assays performed with different monovalent ions revealed similar patterns of inhibition of triplex formation and concomitant increase in self-aggregating products, depending on ion concentration and ion radius (39). The closer in character ions are to K⁺, the more inhibitory they turn out to be. Another in vitro study involving the purine motif produced inhibition of triplex formation by potassium without effects on dissociation rates. Inhibition in this case was apparently not due to the shuttling of oligos into G-quartet formation (40). Pyrimidine TFOs are limited by the requirement of cytosine protonation needed for hydrogen bonding to the duplex. The implication of this proton requirement is that unmodified pyrimidine TFOs must bind under nonphysiological acidic pH.

All motifs require divalent metal cations to neutralize charge repulsion between backbones to allow not only triplex formation but also triplex maintenance. These divalent cations must not be Ni²⁺ or Cd²⁺, which bind to the bases and are detrimental to hydrogen bonding (41). Polyamine compounds such as spermine or spermidine can also serve the function of masking backbone charge (42, 43). At a 500-μM concentration, spermine doubles the association rate at low ion concentration and at neutral pH (42). Conducive to intracellular formation, polyamines can be found in the millimolar range in eukaryotic cells.

VI. Improvements in TFO Chemistry

In order to optimize triplex formation, a number of innovations in TFO chemistry have been developed and tested. Intercalators such as coralyne have been reported to improve triplex stability and function (44). Intercalators have been conjugated to either the 5′ or 3′ ends of oligos for the purpose of anchoring the TFO once the triplex has been formed (45). Intercalators placed in the middle of a TFO have overcome destabilizing mismatches (46). The evolution of intercalator design has led to the advent of a five aromatic ring system, derived from benzo[j]quinol[3,4-b]quinolazoline, which has a preference for intercalating in triple helices (47).

A major limitation of triplex technology that is steadily being overcome is the need for a sufficiently long stretch of purines in the target duplex. The target sequence range has been considerably broadened through the use of base analogs or through the trick of strand crossing. 4-(3-Benzamidophenyl)imidazole allows the binding, through van der Waals interactions, with inversions otherwise destabilizing for the pyrimidine TFOs (48). In addition, interruptions in a purine motif can be skipped by using azo nucleosides which avoid steric hindrance and yet provide base-stacking stability (49). (See also Ref. 50; and for a discussion of targeting inversions, see Ref. 51.) Short purine runs neighboring short pyrimidine runs can be targeted if complex oligos are designed. Purine stretches in both duplex strands can become bound by short oligo components held together by flexible linkers. In certain sequence contexts 5′-pyrimidine-purine-3′ constructs are twice as effective at triplex formation as 5′-purine-pyrimidine-3′ constructs (52). Assisted targeting has also been tried. Mixed sequences have been targeted using an artificial third strand/recA filament. The use of this filament is not expected to result in a formation like a TFO mediated triplex (44).

The pyrimidine dependence on low pH has been circumvented primarily through the use of analogs. If the cytosine on the third strand is methylated at the 5 position, triplex formation can occur at physiological pH (53, 54). Because a run of five methylcytidines can lead to lower triplex stability, however, other replacement analogs—both purine and pyrimidine—have been developed, including the purine analog 8-oxo-2′-deoxyadenosine (55).

The tendency of purine strands to form undesirable secondary structures has been combated by using analogs and by engineering extended secondary structures into the TFOs. In one study the inhibitory effects of high potassium concentrations on purine-motif triplex formation was eliminated by the use of 7-deazaxanthine without a compromise in K⁺ values. The degree of K⁺ resistance using this compound may depend on sequence context (56). Resistance to potassium up to a concentration of 200 mM is possible through the use of 6-thioguanine. Sulfur's larger radius and reduced electronegativity ostensibly produce the steric hindrance and lowered affinity for potassium necessary for prevention of the rise of G quartets (57). Ironically, creation of partially self-complementary oligos may provide protection against G-quartet formation. Such secondary structures would still leave a stretch of unpaired nucleotides which could initiate triplex formation (58).

Many types of chemical substitutions, including changes in backbone linkages, backbone components, and base types, as well as the attachment of positively charged molecules, have been shown to lower repulsions between the
backbones and increase nuclease resistance. Phosphorothioates, which have a nonbridging oxygen substituted with a sulfur, have a much prolonged half-life. Phosphoramidate backbones, which contain an amino group joining the phosphorus atom to the 3' position, have been shown in vitro to bind better to duplex than phosphodiester molecules in the pyrimidine but not the purine motif (59). Morpholino oligonucleotides, which are neutral in charge, contain phosphoramidate linkages, and have morpholine rings instead of deoxyribose, have been shown to be superior in binding compared to phosphoramide oligos in the absence of Mg$^{2+}$ (60). The analog 5-(1-propynyl)-2'-deoxyuridine has also been shown to decrease the need for divalent ions, possibly by virtue of increased stability through improved stacking interactions (61). Levels of Mg$^{2+}$ as well as other cationic molecules have been rendered irrelevant by the conjugation of spermine onto poly(pyrimidine oligos) (62).

### VII. TFOs as Molecular Tools

Because the chemistry of TFOs allows the attachment of various non-nucleic acid molecules, TFOs have been used as targeting vehicles. Because TFOs confer sequence specificity, modifying and cleaving enzymes can have their sites of action restricted. Such sequentially acting oligo/enzyme compounds have demonstrated both the sequence specificity of TFOs and the soundness of using such linked macromolecules to focus protein action. The ability to produce predicted cleaving events offered hope for the development of a unique mapping tool and for a way of expediting the isolation of desired regions. Also, TFOs may be good tools for the study of basic biological processes. The generation of a strand break by other means has already resulted in significant information about recombination (63).

Through a number of means, desired cleavage events, have been achieved using TFOs. EDTA-Fe tethered to a TFO produces strand-breaking free radicals (64). Pyrimidines tethered to phenanthroline in the presence of copper ions and a reducing agent likewise has produced specific double-stranded breaks (65). Double-stranded breaks can also be caused by isotopic radiation, as has been demonstrated by the appending of a 5'-[125]dCMP onto an oligo, whose binding to a plasmid followed by transfection into cells led to a 10,000-fold induction of mutagenesis (66). Also, the duplex/triplex junctions are known to be excellent substrates for photoendonucleases, at least in vitro (67). Cleavage of a single-stranded DNA molecule via a fusion protein containing the active site for a nuclease is also possible (68). TFO coverage of target regions can block cutting by restriction enzymes. Indeed, the restriction enzyme protection assay is an accepted test for triplex formation. TFOs can block methylation sites, thereby allowing restriction enzyme-mediated isolation of designated sections of genomic DNA (69).

The specificity of action of a TFO cleaving tool was established in a paper by Posvic et al. in 1992. They achieved nonenzymatic cleavage by using two TFOs with appended N-bromoacetyl electrophile moieties that flank a target region. These TFOs were constructed so as to alkylate guanines two base pairs from the 5' end of each TFO. After depuration with piperidine, a double-stranded break that left ligatable overhangs occurred in a single site on a 4-kb plasmid or on a 340-kb yeast chromosome. The efficiency was calculated to be around 85% (70).

The ability of TFOs to direct covalent modification in vitro has been repeatedly demonstrated. Alkylation of adenines at the N3 position in the minor groove has been achieved via a tethered 5-methylcytoplencyclopropyloolidole residue (71). To increase success rates, intercalating agents have been used in linkers to help thread minor-groove acting agents to their site of action (72). Alkylation of the N7 position of guanine in the major groove has been accomplished by the attachment of a chlorambucil to purine TFOs. Under conditions not favorable to triplex formation, no such modification occurred (44).

The intercalator psoralen has also been guided by TFOs to preferred sites. Psoralen is a planar tricyclic photocreativatable DNA intercalator that can crosslink DNA by forming a covalent bridge between two thymines on opposite DNA duplex strands (73). Psoralen can be linked to the 5' or 3' ends of oligos via carbon linkers (Fig. 2). If TFO binding sites are located right next to 5'-TpA-3'

---

**Fig. 2.** Strategy for triple-helix formation with psAG10. The sequence of the psoralen-linked oligonucleotide, psAG10, is shown positioned opposite the target sequence binding site for triplex formation in the supF gene, an E. coli amber suppressor tRNA. The psoralen moiety tethered at the 5' terminus was designed to react with the thymines at bps 166–167. Reprinted with permission from F. P. Gasparro et al., Site specific targeting of psoralen photodadducts with a triple helix forming oligonucleotide, characterization of psoralen monoadduct and crosslink formation, Nucleic Acids Res. 22(14), 2845–2852 (1994).
dinucleotide sequences, psoralen can be incorporated at that site at a high frequency by a psoralen–TF0 construct upon UVA irradiation. Substantial improvements in the targeting of mutations have been reported using psoralen TFOs (74). In vitro studies have yielded up to 90% adduct formation and, more specifically, about 80% crosslink formation using TFOs conjugated with psoralen (75). The degree of freedom of action of the psoralen is determined by the length of the linker (76). For the purpose of studying the effect of a particular psoralen adduct in the absence of the delivering oligo, disulfide linkers that can be reduced have been designed so that the oligo is released from the site (77).

One of the first papers to demonstrate such directed action and to posit practical ramifications was published in 1991 (78). Targeted intercalation next to a reporter gene via this method was subsequently shown. Gel shift assays and HPLC analysis confirmed the preference for monoadduct formation on the purine strand in a 4:5'-furanside monoadduct versus a 3,4-pyroneside monoadduct formation (79).

VIII. The Chromatin Barrier

The ease with which a TFO can gain access to a chromatinized target sequence is a matter open to debate. On this issue the in vitro and in vivo evidence seem to be contradictory. If 22-mer triplex complexes, but not 10-mer, are preformed on linearized DNA fragments highly receptive to both TFOs and nucleosomes, footprinting assays reveal an inability to reconstitute nucleosomes. Conversely, if the nucleosomes are preformed and the DNA then exposed to 22-mer TFOs, no displacement of the histone assembly is seen unless performed under destabilizing high salt conditions (80). From hydroxyl-radical cleavage patterns derived in another study, it was discovered that both parallel and antiparallel TFO binding tended to be restricted to parts of the target DNA that were in weakest contact with histone proteins (81).

These results notwithstanding, mutagenesis and crosslinking data gathered using psoralen-conjugated TFOs point to the accessibility of chromatinized targets. Polypurine tracts of HIV-1 provirus integrated in mammalian cells permeabilized by digitonin and incubated with special binding buffer have been crosslinked by psoralen–TFOs. Comparison between different cell lines with different sites of viral incorporation has revealed the importance of target location for the frequency of success (82). A native gene, an allele of the MHCII locus, in a genomic prep was covalently modified using a chlorambucil TFO with an 80% efficiency at 0.5 μM (44). In 1998, the alkylation of an endogenous target was reported. A 12-mer purine oligo linked to a nitrogen mustard entered streptolysin O-treated HT-29 adenocarcinoma cells and successfully modified a chemokine receptor target, the CCR5 HIV receptor gene, as determined by ligation-mediated PCR (83). A subsequent report has cautioned that ligation-mediated PCR has been found to produce artifacts if precautions are not taken (84).

Other work using mutagenesis as an assay also demonstrated TFO targeting of chromosomal sites. Using a mouse fibroblast cell line containing multiple copies of a supF reporter gene optimized for triplex formation by the insertion of a purpurine sequence, Vazquez et al. were able to detect localized mutagenesis. Targeted mutagenesis by a TFO molecule was detected by the isolation of genomic DNA, packaging the supF containing lambda phage vector, and infecting bacteria. The lawn of bacteria on IPTG and X Gal containing plates revealed either blue or white plaques depending upon the ability of the packaged vector DNA to suppress an amber mutation in the lacZ gene of the bacteria and so to produce a colored metabolite. In a forward assay the supF gene would be functional until TFO-induced mutagenesis prevented the proper tRNA folding, ultimately causing the production of white plaques (Fig. 3). Using this system and relying upon passive uptake of oligos at a 2 μM concentration led to a 6- to 10-fold increase in mutagenesis. Sequence analysis revealed mostly insertions and deletions around the triplex binding site (85).

The accessibility issue has been addressed in other work as well. A collagenase gene in human fibroblast cells was crosslinked in situ at two sites by psoralen TFOs that were lipofected into the cells at a concentration of 250 nM. Confirmation was by single-strand ligation PCR. This method involves rescuing the genomic DNA, cleaving around the TFO binding site, extending a primer up to the point of blockage, ligating a known sequence to the end of the PCR product, then PCRing with primers complementary to regions just inside the first primer and to the ligated end, and checking for abbreviated products. Interestingly, the addition of the transcription inducer phorbol ester failed to improve targeting frequency as judged by an absence of an increased frequency of abbreviated primer extension products (86).

The ultimate test of targeting ability was carried out in live mice with the use of unsopralenal 3'-end modified TFOs. A 5-fold induction of mutagenesis was seen in the supFG1 gene in these transgenic mice, which were given intraperitoneal injections of 1 mg of oligo per day for 5 consecutive days. At the time of sacrifice, all major tissues were examined and all except brain were found to contain approximately similar mutation rates. Controls included scrambled oligos, an untargeted control reporter gene, and the supposedly inaccessible brain tissue. In all cases, as expected, mutation rates were at background levels (87).

How can the in vitro and in vivo results be reconciled? Both types of studies involved similar kinds of TFOs, with similar lengths and binding affinity. Like the nucleosome reconstitution studies, the mouse data contained nonpsoralenated TFOs. It appears that the in vitro studies must not be adequate reflections of the
in vitro situation, which is more dynamic and where phasing is variable. Might the presence of H1 or other cell components absent in the in vitro studies make an unexpected difference? Is the confinement of the in vitro studies to short stretches of DNA too artificial? As for the absence of a difference in targeting efficiency in the presence of a transcriptional activator, perhaps the particular region studied under the given circumstance was for the applied concentration of oligonucleotides maximally accessible.

**IX. TFOs as Antigene Agents**

Unlike antisense strategies, which rely upon the successful elimination of numerous messenger molecules, TFOs bear the promise of shutting down the very source of those messages. In the late 1980s and early 1990s, a series of in vitro studies was performed that examined the ability of TFOs to block transcription initiation or elongation. As determined by footprinting assays, TFOs without linked binding agents occluded polymerase binding to a bacteriophage artificial operator sequence (an inserted triplex-amenable polypurine sequence) (88). Another study showed interference with transcription to be dependent on the position and number of TFO binding sites (89). Hope for increasing the number of possible endogenous targets comes partly from the finding that target regions for the purpose of inhibition may also be located away from the proximal promoter region. A 13-bp sequence found in the coding region of the bla gene of the Tn3 transposon has been bound and functional mRNA production prevented. Transcription could be resumed if the TFO was melted off the template (90). Longer TFOs, and hence stronger binders, have been found to be better inhibitors of elongation. Enhancers of transcription have also been occluded. The transcription factor Sp1 has been prevented from binding to its normal site by prior triplex formation (91).

The demonstration of TFO-mediated RNA level reduction in intact cells was soon forthcoming. Progesterone response elements residing in the tyrosine aminotransferase gene were targeted by a mixed purine/pyrimidine TFO which was linked to cholesterol. Monkey kidney CV-1 cells were transfected with plasmids bearing the target region upstream of the CAT reporter gene. Following incubation with these TFOs at a concentration of 20 μM and then with progesterone, CAT activity was reduced 50% (92). The polypurine tract of HIV-1 placed in a plasmid in the 5' untranslated region of a luciferase reporter gene has been targeted by phosphoramide oligos. Transcription elongation was blocked if the target was episomal or integrated in the genome of HeLa cells. Inhibition of the production of functional luciferase was seen at the single cell level (93). Overall protein reduction was around 50%. Controls for this work included
scrambled oligos and mismatched target sequences that expressed comparable basal levels of luciferase mRNA, and hence should have been equally accessible. As further indication that the effect was triplex-mediated, the effectiveness was improved by the inclusion of triplex-stabilizing intercalators (93).

The attainment of a desired phenotypic effect was also observed in experiments targeting the C-myc oncogene. A 10 μM concentration of a 22-mer G/T oligo was able to reduce the RNA and protein levels within 24 h to 50%. Flow cytometry indicated a ~20% increase in the amount of transfected cells trapped in S phase. At such a dose a 5- to 6-fold increase in leukemia cell mortality was seen after 4 days (94). In another example, tumor progression in mice was slowed by the targeting of the promoter of the IGF-1 receptor (95).

X. TFO-Mediated Upregulation of Gene Expression

The ability to modulate a chosen gene’s expression would be of benefit both to basic research and to medicine. For research purposes, it would be useful to ectopically turn on genes to discover phenotypes that may result from subtle overexpression. For instance, developmental biology questions dealing with the finetuning of embryonic stage genes could then be addressed. Current methods that involve transfection with additional copies of the gene, even if under the control of inducible promoters, are easy but relatively crude. Copies that randomly incorporate themselves into chromosomes may be silenced or have negative effects on neighboring genes. For therapeutic purposes, it would be ideal to be able to stimulate the production of gene products whose absence leads to illness. TFOs could potentially serve as weapons in the arsenal against cancer if they could switch on genes that promote apoptosis. Alternative strategies for increasing gene expression often lead to the upregulation of an entire class or classes of genes rather than single, specific genes. For example, some natural or artificially synthesized chemicals such as hormones can directly or indirectly upregulate myriad genes responsible for proliferation. Extreme treatments of cells, such as heat shocking or serum starvation triggers, heighten transcription of many survival genes.

The means by which triplex-forming molecules could turn on gene expression include nucleosome repositioning, mutation of repressor binding sites, formation of artificial transcription bubbles, recruitment of transcription factors, and physically dragging transcription factor–activating domains to promoter regions. The basal transcriptional apparatus is known to compete with nucleosomes. If relieved of histones, a template is more accessible and more likely to be traversed by a polymerase. The binding of a TFO to a chromatinized target appears to be in competition with histones. Once bound, however, a TFO might advantageously change the phasing and so alleviate nucleosome mediated repression. The ability of preformed triplexes to restrict nucleosome placement has been shown in vitro (96).

A therapeutic goal for researchers involved with sickle cell anemia and thalassemia disorders is the upregulation of γ-globin, which is normally expressed only during early stages of development. β-Globin is mutated in patients suffering from either disorder, but amelioration of symptoms can occur when γ-globin expression is elevated. An 11-mer purine oligo with a tethered psoralen has been designed to crosslink a site within the γ-globin gene promoter. When the target is located in a plasmid subsequently transfected into human fibroblast cells, resultant mutations within the promoter cause a 4-fold increase in γ-globin mRNA. In vitro binding assays showed that Oct 1 repressor binding to the promoter was reduced with such mutations (97). γ-Globin mRNA levels were also increased by the employment of two pyrimidine-motif PNA clamps, which were shown to bind and unwind an upstream region. In vitro transcription assays revealed the start points to be at the TFO binding site as well as at the normal start site (98). The inspiration for this latter work had come from previous experiments with E. coli (99).

A more ambitious strategy involving transcription factor recruitment has also been attempted. A two-domain TFO has been designed with a single-stranded portion for triplex formation and a double-stranded section containing binding sites for the transcription factors SRF and ELK. While footprinting and competition assays indicated triplex formation on a plasmid and sequential transcription factor binding, no evidence for biological activity has been presented (100). An alternative construct in which multiple copies of the activating domain of the herpes simplex virus protein 16 (HSV VP16) were conjugated to a polyurine TFO, a modest upregulation (3- to 4-fold) was seen of a reporter gene situated on a plasmid when the triplex was extracellularly formed (101). Because of large error bars and the lack of a no-oligo control, it is not clear whether the activation represented, at least in part, relief of TFO-produced repression.

In addition to pharmacokinetic and toxicological questions raised by oligonucleotide-based medicines in general, the aim of upregulating a gene’s expression prompts a host of particular concerns. For how long can, and should, such an effect last? Will it require significant basal level expression and/or replication around the target in order for TFOs to bind? Can such TFOs turn on only one gene in isolation from all others, and can it be made to do so in a particular cell type? To address these sorts of questions, gene chip experiments seem appropriate. Secondary effects need to be investigated as well. What will be the response of the cell? Does the TFO directly or indirectly cause the cell to produce countermeasures that may negate the primary stimulation? Is the targeted gene under regulation so that the production of more protein actually leads to repression? Most importantly, does the overexpression of the gene result in the expected phenotype?
XI. TFO-Mediated Mutagenesis

A number of mutagenesis studies from our lab and others have confirmed the ability to target a particular site for psoralen damage using TFOs as targeting reagents. A TFO linked to psoralen was found to produce damage in the supF reporter gene in a concentration- and UVA-dose-dependent manner. In experiments to target the supF gene contained in an SV40 vector, psoralen-TFO damage was targeted in vitro, followed by passage of the vector DNA through monkey COS cells, yielding mutation frequencies in the range of 5–10% (105). Analysis revealed mostly point mutations located at the predicted site of psoralen intercalation at the duplex/triplex junction. Assays requiring intracellular triplex formation have also detected targeted mutagenesis. In 1995, it was shown that treatment of COS cells already containing the SV40 vector with a concentration of 2 μM TFO yielded a mutation frequency of 2.1% (versus a background level of 0.03%). Most of the mutations were T·A to A·T transversions at the TpA site, consistent with the mutagenic activity of psoralen adducts. A low frequency of deletions was also seen (103).

In experiments to study TFO-mediated targeting of a chromosomal gene, mouse fibroblast cell lines containing multiple copies of the supFG1 gene were transfected with psoralen-conjugated purine TFOs designed to bind to a 30-bp polypurine sequence in the gene. The result was an induction of mutations by a factor of at least 6 compared with a scrambled control oligo. However, the types of mutations generated were different from the signature psoralen mutation pattern of T·A to A·T transversions. These mutations were proposed to be due to strand slippage events occurring during triplex-induced repair synthesis. The poly C tract in the target gene would be prone to dislocation and misalignment during repair synthesis, which could lead to the deletions and insertions noted (85).

A recent report on psoralen-TFO mutagenesis in yeast has looked at the impact of backbone chemistry on mutagenesis efficiency. In the reporter system used, the production of mutations at the psoralen target site reverts an ochre mutation and thus restores a ura3 selectable gene. If the triplexes were preformed using phosphodiester, psoralen-linked TFOs and the complex then transfected into ura3(−) cells, reversion rates were on the order of 15%. Even though phosphoramidate oligos have greater affinity for their targets and are better able to resist degrading enzymes (104), their use in this situation led to only about a 1% mutation frequency. The relative efficacy of each was reversed when the mutation was in an endogenous, chromosomal ura3 gene. In this case TFOs with phosphoramidate backbones were 40 times more effective in generating revertants, but at a low absolute frequency (40 × 10⁻⁷). While mutation frequencies seem low, this is in part due to the fact that other psoralen-induced or triplex-induced mutations could not be detected by this assay. For a negative control, an isogenic strain was used that had mismatches only in the TFO binding site but retained the same TpA psoralen target site within the ochre codon (105).

In other efforts to target a chromosomal gene in mammalian cells, the hypoxanthine phosphoribosyl transferase gene (hpert) was knocked out by TFO molecules in CHO cells, allowing survival in selection media. A region around the intron 4-exon 5 boundary was chosen as a third-strand target site for a pyrimidine TFO, with the tethered psoralen directed at a TpA within the splice acceptor region. Induced mutations were detected at a frequency at least 5-fold above background, in the range of 10⁻⁴, when a 10-μM concentration of a 23-mer pyrimidine TFO containing 2′-O-methyl sugar modifications, methylcytosine instead of cytosine, internal intercalators, and a 5′-linked psoralen was electroporated into the cells. Only those oligos possessing either a pyrene or acridine conjugate necessary to improve binding affinity via intercalation were effective. PCR and sequencing analysis confirmed that the mutations were generated at the triplex target site (106).

XII. TFO-Mediated Recombination

Easy, efficient gene replacement is a long-term goal of gene therapy research. The introduction of foreign sequence information usually results in nonspecific incorporations through end joining or nonhomologous recombination events. Inducers of DNA damage, including UV radiation (107), carcinogenic compounds (108), and the crosslinking agent psoralen (109), are known to stimulate recombination events in eukaryotic cells. Despite some discerned sequence preferences, these agents inflict damage in a nearly random manner. Success in gene replacement might then be expected to depend on the development of constructs that are simultaneously sequence-specific and recombination-provoking.

Preliminary results from experiments designed to detect increases in intermolecular recombination due to site-directed psoralen crosslinking yielded modest results. Two SV40 shuttle plasmids, one containing a defective copy of the supF reporter gene bound by a psoralen-TFO and the other containing a wild-type copy but missing the ability to replicate in cells, were transfected into human Jurkat cells. Only if homologous recombination occurred could functional supF (and consequently lacZ) be made in indicator bacteria electroporated with the rescued plasmid. Recombination rates on the order of 0.05% were observed. As a point of comparison, a linearized donor plasmid was also cotransfected as a one control condition. Under these circumstances a 2% recombination rate was obtained. The conclusion of the authors was that psoralen damage was either poorly corrected or at least not frequently corrected by a recombination pathway (110). Caveats include the possibility that the psoralen photoprodut production
in these experiments may have been suboptimal, and that the experiment required the successful transfection of both constructs into each cell, which may have been a low frequency event.

Our group has studied intramolecular recombination provoked by intermolecular triplex formation. For these studies an SV40 shuttle vector was created that contained two tandem mutated copies of the supF reporter gene flanking a triplex binding site but with a slight overlap in the 3' end of the first copy (Fig. 4). When psoralen triplexes were allowed to form on the plasmid prior to transfection into COS cells, recombination occurred 14% of the time relative to a background level of 0.02%. When intracellular triplex formation was attempted, recombination rates were about 0.53% while mutation rates (which could also be measured in the assay) were similar at 0.77%. Recombination always produced a nonparental supF sequence. This was confirmed by observing redistribution of silent marker mutations engineered into the supFG1 genes. Furthermore, one copy was usually preferentially altered, consistent with a gene conversion mechanism. This copy tended to be the one with overlap with the triplex binding site and hence the psoralen damaged copy. Reversion mutations were ruled out as an explanation for the high level of correction because psoralen–TFOs crosslinked onto plasmids that contained only a single mutant copy produced minimal levels of regained function. Through controls it was discovered that the high level of recombination was made possible by the presence of the crosslinks and of the third strand (111).

In a subsequent paper our lab has provided evidence of TFO-induced recombination in the absence of psoralen. The same system described above was used, and with the triplexes intracellularly formed in COS cells. A 5-fold stimulation of intramolecular recombination was witnessed without psoralen, as opposed to a ~20-fold stimulation when psoralen accompanied the TFO. The experiments were also carried out in XPA-deficient cell lines, which lack the XPA damage recognition factor that can initiate nucleotide excision repair (NER). The results indicated that NER was essential for the triplex-stimulated recombination, indicating a requirement for NER-mediated strand breaks for the triplex-induced recombination (112).

Our group has also obtained encouraging data that suggest not only that recombination can be induced by a TFO but also that a bifunctional oligonucleotide can provide the sequence information used by cellular machinery. A supFG1 reporter gene in an SV40 shuttle vector was targeted with an oligonucleotide designed to have two domains. A triple-helix forming single-stranded 30-nt portion (AG30) was tethered to a short (40 bp or nt) donor fragment, either double- or single-stranded, designed to provide the homologous sequence information, except for a single base pair difference at the position to be changed (Fig. 5). $K_4$ values were minimally altered by the burden of an attached 40-bp duplex via a flexible linker. The theory behind the design was that the formation of a triple helix by the TFO portion might provoke cellular DNA repair or recombination processes while the donor domain might be used by the machinery as a template for information transfer. TFO-donor conjugates in both purine and pyrimidine motifs were tested for the ability to generate single or multiple base changes in the target gene, detectable by either a forward mutation assay or a reversion assay. When the AG30/donor DNA duplex was bound onto the plasmid before transfection into monkey COS 7 cells, reversion frequencies of ~1% were detected. When the targeting was mediated by intracellular triplex formation, a successful correction frequency of 0.04%, or a 50-fold improvement over background, was obtained (113). The method of oligo transfection affects the levels of success, with a 10-fold improvement to 0.5% with cationic lipids instead of electroporation as the transfection method (unpublished data). When the TFO domain and the donor domain
bind to the polypurine site, recombination resulting in a functional TK gene was detectable by selection in hypoxanthine–aminopterin–thymidine (HAT) medium. Microinjection of about 70,000 oligonucleotide molecules per cell increased the incidence of HAT-resistant colonies to 1%, or 2500 times the background level (versus the lesser 6-fold increase when the oligos were delivered by cationic lipids) (114). Southern analysis of the genomic DNA from the HAT-resistant colonies revealed that functional copies of the TK gene were produced by conservative gene conversion events (114).

XIII. TFO-Directed Sequence Change

The ability to actually change a DNA sequence in a directed fashion would open up new gene therapy possibilities. Current transgenic techniques rely upon a low rate of successful homologous recombination on the order of one in a thousand specific events and necessitate positive and negative selection of cells in culture.

TFOs, because they can induce predictable mutations, present new options. An early attempt used a TFO to target the mutagen psoralen to a specific base pair in the supF gene in an intact lambda genome. After crosslinking and passage through bacteria, mutations arose at levels at least 100 times that seen in an untargeted gene. This report found that slightly more than half of the mutations were T-to-A transversions at the site of psoralen intercalation (115). Similar findings were obtained when looking at intracellular TFO-directed mutagenesis on a shuttle vector in COS cells, with mutation frequencies of up to 2%, as noted above (103).

Following upon this observation, Fresco and colleagues have focused on the mutation in the β-globin gene associated with sickle cell anemia. Sickle cell anemia is caused by a single A: T to T: A transversion. Unfortunately, the region in the β-globin gene near the sickle mutation lacks an uninterrupted polypurine sequence. To overcome this, the Fresco study used modified oligos that possessed not only base analogs (5-methylcytosine and 5-propynyluracil) but also components that allowed binding to short purine stretches on alternate strands, as well as the ability to bind one strand of the target by Watson-Crick pairing. Specific binding and photoproduction formation at the target base pair within a plasmid containing the target gene were clearly detected (116). However, the intracellular activity of this TFO remains to be tested.

XIV. Repair Systems Implicated in TFO-Induced DNA Alterations

The phenomena of TFO-induced mutagenesis, recombination, or directed sequence change are most likely the consequence of at least two events: the
production of an unusual structure on the DNA which is treated as damage, and the actual execution of the damage recognition and repair process(es). The full details of both events are currently unknown, although hints as to the nature of the latter event are emerging and offer insight into the probable nature of the first. Definitive answers regarding both events would help in the design of the next generation of TFO agents.

TFOs, even without tethered mutagens, have been shown to provoke mutagenesis (85) and recombination (112). In the absence of conjugated moieties, it is unclear what form the damage might take. Of the common types of DNA alterations known to be recognized by a cell (nicks, double-stranded breaks, base damage, pyrimidine dimers, mismatches, and bulky adducts), it is most likely that the last of these explains how the triplex is sensed. A bulky adduct is a single- or multinucleotide generic obstruction that can alter the structure of the double helix, which fits the description of a TFO-induced triplex. Replication or transcription may be blocked because of such an aberrant structure, or the altered helix structure itself could trigger repair. Several studies have demonstrated the ability of TFOs to hinder RNA polymerase progression, raising the possibility that triplex formation could be resolved in some instances by transcription-coupled repair.

As for the repair systems responsible for recognizing triplexes, evidence points toward involvement of the NER pathway. In an in vitro assay for repair in HeLa cell extracts, high-affinity triplex formation could induce general repair synthesis on a plasmid substrate (117). Comparisons have been made between the mutagenic effects of TFOs in normal human fibroblast cells and in those deficient in XPA, a NER recognition protein known to bind to junctions between unwound and duplex DNA. Both NER and its related pathway, transcription-coupled repair, have been implicated in the recognition of triplex damage caused by TFOs. Intracellular triplex formation in COS cells on a supFG1 reporter plasmid led to induced mutagenesis at and around the triplex site, mostly one- or multiple-point mutations and some large deletions. Cells deficient in either XPA or CSB, a protein essential for transcription-coupled repair (TCR), had reduced mutagenesis, indicating a role for both NER and TCR in the induced mutagenesis.

Psoralen--TFOs not only can stimulate repair, but, depending on their length, can also inhibit or influence the pattern of repair and mutagenesis. Reduction in the level of excision products was seen in an in vitro repair assay using HeLa cell extracts when the psoralen oligo bound was 30 nucleotides long, but not when it was 10. In live COS cell experiments, the respective mutation frequencies caused by the 30-mer versus the 10-mer were 2.8% and 5.2%, respectively, when the triplex was preformed before transfecting the plasmid. Analysis of the mutations revealed that the mutations caused by the psoralen 30-mer were shifted one base relative to the position of the transversions resulting from the

---

**Fig. 6.** Model for psoralen adduct repair (A) and bypass replication (B) in the presence of a triple helix. The stick diagrams indicate the potential repair pathways for oligonucleotide-directed monoadducts and crosslinks. The psoralen-conjugated oligonucleotides are represented by the smaller third strands in each diagram, being connected to the duplex by either one line (monoadduct) or two lines (crosslink). The small arrows mark predicted sites of endonuclease incisions based on the reported properties of the nucleotide excision repair complex in mammalian cells. Displaced arrows are meant to suggest possible inhibition of the endonuclease activity by the third strand. DNA synthesis, either as a component of the repair reaction or in trans-ligation bypass replication, is represented by the dashed lines. Reprinted with permission from C. Wang and P. M. Glazer, Altered repair of targeted psoralen photodadducts in the context of an oligonucleotide-mediated triplex helix, *J. Biol. Chem. 270*(36): 22590-22591 (1995).
presence of the 10-mer in association with the psoralen conjugate. This may be the result of prevention by the 30-mer of the incision of the preferred strand [118]. To explain the origin of the mutations, a model for repair of these crosslinks has been offered (Fig. 6).

Other work has also shown an effect of a triple helix on repair. A similar length-dependent inhibition was obtained with a HeLa cell extract experiment that used a Southern blotting technique to examine the identities of excision products [119]. Here again, endonuclease activity was prevented in the presence of the TFO but not when the TFO was removed before exposure to the extracts. To further study the influences of triplexes on repair processes in living cells, a luciferase assay was set up in HeLa cells whereby expression was dependent on repair of the crosslinked/triplex region. Psoralen in the absence of a TFO was ostensibly removed at a 6- to 7-fold higher frequency than psoralen linked to a TFO [119].

While many indicators seem to point to NER as essential to mutagenesis, is it actually the immediate cause of the misincorporations? The answer seems to be No, and the culprit is likely to be an error-prone repair or lesion bypass system. Yeast knockouts experiments furnish persuasive evidence. Plasmids containing a selectable marker crosslinked in vitro by a psoralen-TFO and then transformed into yeast have low survival and a 1% mutation frequency, mostly base substitutions and single base insertions. When the rad18 gene is rendered nonfunctional, and so the error-prone repair pathway is compromised, mutagenesis falls to background levels. The same result is not true when rad1, responsible for the incisions during NER, is missing. In this case, mutation increases in screens designed to detect insertions but decreases in screens better able to detect substitutions [120]. Recombinational repair appears to process triplex lesions in an error-free manner. Elimination of the rad51 gene, which encodes the strand exchange protein, raises mutation levels [120]. In human cells, psoralen-TFO crosslinks were found to cause an increased level of mutation in cells from patients with xeroderma pigmentosum variant (XPV) [77]. XPV cells are now known to be defective in the human rad30 homolog, and thus have abnormality in translesion bypass polymerase activity [121, 122].

**XV. Conclusion**

Triplex technology is beginning to deliver on its promise of allowing the manipulation of gene expression and alteration of gene sequences. While still burdened by concerns about efficient delivery and polypurine target site restriction, improvements in TFO chemistry and transection methods have greatly enhanced the prospects for ultimate success. It remains to be seen whether other sequence-specific agents are versatile and effective in modifying genomes.
GENE TARGETING VIA TRIPLE-HELIX FORMATION

Searching New Targets for Anticancer Drug Design: The Families of Ras and Rho GTPases and Their Effectors

SALVADOR AZNAR AND JUAN CARLOS LACLÁN
Instituto de Investigaciones Biomédicas CSIC, 28029 Madrid, Spain

I. Rho Proteins and Their Effectors ........................................ 196
   A. Wiskott–Aldrich Syndrome Protein (WASP) .......................... 198
   B. IQGAP ........................................................................ 199
   C. ACK .................................................................. 201
   D. p21-Activated Kinase (PAK) .......................................... 201
   E. ROCK Family: Multifunctional Effectors .......................... 204
   F. Phospholipase D (PLD) ............................................... 205
   G. Protein Kinase N (PKN) ............................................ 207
II. Ras Proteins and Their Effectors ......................................... 209
   A. Raf Kinase Pathway ...................................................... 211
   B. Phosphatidylinositol-3-OH Kinase (PI3K) ..................... 212
   C. RalGDS Family ........................................................... 214
III. Pharmacological Approaches to Reverting Transformation by GTPases of the Ras and Rho Families ........................................ 215
   A. ROCK Inhibitors with Antitumor and Antiangiogenic Activity 216
   B. PLD as a Target for Cancer Treatment ............................ 216
   C. Choline Kinase Inhibitors Have in Vivo Antitumor Activity 217
   D. Inhibition of Raf Kinase ............................................... 218
   E. Inhibition of Ras and Rho Farnesylation .......................... 219
   F. Other Potential Targets for Drug Design ........................ 221
IV. Future Perspectives ................................................................. 223
References ........................................................................... 223

The Ras superfamily of low-molecular-weight GTPases are proteins that, in response to diverse stimuli, control key cellular processes such as cell growth and development, apoptosis, lipid metabolism, cytoarchitecture, membrane trafficking, and transcriptional regulation. More than 100 genes of this superfamily grouped in six subfamilies have been described so far, pointing to the complexities and specificities of their cellular functions. Dysregulation of members of at least two of these families (the Ras and the Rho families) is involved in the

1Corresponding author. Telephone +34-91.585.4607; fax +34-91.585.4606; E-mail: jclalan@ibb.csic.es.