Altered Repair of Targeted Psoralen Photoadducts in the Context of an Oligonucleotide-mediated Triple Helix*

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Oligonucleotides can bind as third strands of DNA in a sequence-specific manner to form triple helices. Psoralen-conjugated, triple-forming oligonucleotides (TFOs) have been used for the site-specific modification of DNA to inhibit transcription and to target mutations to selected genes. Such strategies, however, must take into account the ability of the cell to repair the triple-directed lesion. We report experiments showing that the pattern of mutations produced by triplex-targeted psoralen adducts in an SV40 shuttle vector in monkey COS cells can be influenced by the associated third strand. Mutations induced by psoralen adducts in the context of a TFO of length 10 were the same as those generated by isolated adducts but were found to be different from those generated in the presence of a TFO of length 30 at the same target site. In complementary experiments, HeLa whole cell extracts were used to directly assess repair of the TFO-directed psoralen adducts in vitro. Excision of the damaged DNA was inhibited in the context of the 30-mer TFO, but not the 10-mer. These results suggest that an extended triple helix of length 30, which exceeds the typical size of the nucleotide excision repair patch in mammalian cells, can alter repair of an associated psoralen adduct. We present a model correlating these results and proposing that the incision steps in nucleotide excision repair in mammalian cells can be blocked by the presence of a third strand of sufficient length and binding affinity, thereby changing the pattern of mutations. These results may have implications for the use of triplex-forming oligonucleotides for genetic manipulation, and they may lead to the use of such oligonucleotides as tools to probe DNA repair pathways.

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*The abbreviations used are: TFO, triplex-forming oligonucleotide; NER, nucleotide excision repair; HPLC, high performance liquid chromatography.
versus 30 generate different mutational spectra at the same target site, with mutagenesis in the context of the 10-mer being similar to that produced by a psoralen adduct alone. In these experiments, we also find a difference between the spectrum of mutations generated by psoralen monoadducts and cross-links targeted to the same intercalation site, a result which has additional implications for models of interstrand cross-link repair. Furthermore, using human cell extracts to study the repair of the shuttle vector-psoralen-TFO substrate in vitro, we show that a triple helix of sufficient length and binding affinity can inhibit excision of the damaged DNA. We propose a model correlating these results and suggesting that the incision steps in NER can be blocked by the presence of a third strand.

MATERIALS AND METHODS

Oligonucleotides and Vectors—Psoralen-linked oligonucleotides were obtained from Oligos Etc. (Wilsonville, OR) or were synthesized by J. Flory of the W. M. Keck Biotechnology Resource Center at Yale using materials from Glen Research (Sterling, VA). The psoralen is incorporated into the oligonucleotide synthesis as a psoralen phosphoramidite, resulting in oligonucleotides linked at their 5′ end via a two-carbon linker arm to 4′-hydroxyethyl-4,5,8-trimethylpsoralen, either psogoAG10 or psogoAG30 (Fig. 1). PsogoS-S-AG10, containing a disulfide bond in the linker arm connecting the psoralen to the oligonucleotide, was synthesized by incorporation of a thiol modifier (S-S) phosphoramidite (Glen Research) into the automated synthesis just prior to the addition of the 5′-psoralen, yielding an oligonucleotide with the same sequence as psogoAG10, but with the psoralen and the oligonucleotide separable by disulfide bond reduction. To preserve the disulfide linker in the synthesis, modification of the oxidation step was required, as directed by Glen Research.

The SV40 shuttle vector, pSupFG1, was previously constructed from the vector pSP189 to contain a modified psF amber suppressor tRNA gene (spFG1) as a mutation reporter gene incorporating a 30-base pair polyuridine/polyuridine site amenable to triple helix formation (13).

Triplex Formation and Targeted Photoadduct Formation—The SV40 vector DNA at 80 nM was incubated with either psogoAG10 or psogoAG30 at 1 μM and irradiated with either long wavelength UV light (UVA, 320–400 nm) at a dose of 1.8 J/cm^2 or with visible light (447 nm) at a dose of 11.7 J/cm^2. Irradiation was delivered using 365 and 447 lamps obtained from Southern New England Ultraviolet, Inc. (Branford, CT). A window glass filter was used to eliminate UVB radiation. A radiometer equipped with filters for UVA and visible light (obtained from International Light, Newburyport, MA) was used to measure the lamp output (typical UVA irradiance of 5–7 milliwatts/cm^2 at 320–400 nm). The formation of specific photoadducts (monoadducts and cross-links) was monitored by gel mobility shift assays and confirmed by HPLC analysis (12).

In the case of psogo-S-S-AG10, the irradiated mixture was further incubated with 50 mm dithiothreitol at 55 °C for 3–4 h to release the oligonucleotide by disulfide bond reduction. The detached oligonucleotide was separated from the duplex by heating the mixture at 65 °C for 5 min and removed by filtration through a Centricron-100 filter (Amicon, Beverly, MA), yielding pSupFG1 containing a site-specific psoralen adduct with an associated oligonucleotide.

Successful detachment of the oligonucleotide from the targeted psoralen adduct was monitored by end-labeling psogo-S-S-AG10 with [α-32P]deoxy-ATP (Amersham Corp.) and terminal transferase. The sample was analyzed before and after dithiothreitol treatment and Centricron-100 filtration by denaturing gel electrophoresis and autoradiography.

Shuttle Vector Mutagenesis—The plasmid-psoralen-oligonucleotide complexes were transfected into monkey COS-7 cells (ATCC 1651-CRL) via electroporation as described previously (13). Following 48 h to allow repair and replication, the SV40 vector DNA was harvested from the COS cells using a modified alkaline lysis procedure, as described (13). The harvested vector DNA was used to transform either E. coli strain SY204 (lacZ125 amber suppressor tRNA gene, respectively, in the anti-parallel triple helix motif (3, 26). Triplex formation by either oligonucleotide positions the psoralen conjugate for intercalation between base pairs 166 and 167. In previous work, we have shown that psogoAG10 and psogoAG30 bind to the target site in

In Vitro Repair Assays—HeLa cell-free extracts were prepared as described previously (24). Oligonucleotides psogoAG10 and psogoAG30 were 3′ end-labeled using terminal transferase and [α-32P]deoxy-ATP (Amersham). Triplex formation with the target plasmid DNA (pSupFG1) was performed as described above. Following triplex formation and photoadduct generation, the majority of the unbound oligonucleotide was removed from the sample by dilution and filtration using Centricron-100 filters (Amicon, Beverly, MA).

Repair reactions were carried out essentially as described (25), with slight modifications. Plasmid-psoralen-oligonucleotide DNAs at 2 × 10^9–10^9 M were incubated for 3 h at 30 °C in the HeLa cell extracts containing 10–15 μg/ml protein and supplemented as described (25). The DNA was extracted with phenol/chloroform and concentrated by ethanol precipitation. The samples were diluted 10-fold with formamide, boiled for 5 min, and analyzed by polyacrylamide gel electrophoresis in an 8% gel containing 7 M urea, followed by autoradiography.

RESULTS

The experimental protocol used to study mutagenesis in mammalian cells induced by triple helix-directed psoralen photoadducts is diagrammed in Fig. 1. The assay is based on the use of an SV40-based shuttle vector, pSupFG1 (13). This vector contains the supFG1 gene, an amber suppressor tyrosine tRNA gene of E. coli, as a mutation reporter gene, along with both the SV40 and pBR327 origins of replication. The supFG1 gene is a functional derivative of the supF gene that has been modified by the incorporation of a 30-base pair polyuridine/polyuridine site for triple helix formation at its 3′ end (13). Psoralen-conjugated oligonucleotides psogoAG10 and psogoAG30 were designed to bind as third strands to base pairs 167–176 and base pairs 167–196 of the supFG1 gene, respectively, in the anti-parallel triple helix motif (3, 26). Triplex formation by either oligonucleotide positions the psoralen conjugate for intercalation between base pairs 166 and 167. In previous work, we have shown that psogoAG10 and psogoAG30 bind to the target site in

FIG. 1. Experimental strategy to study mutagenesis mediated by triple helix-directed psoralen adducts. The triplex-forming oligonucleotides, either psogoAG10 or psogoAG30, are shown directly above their target sequences in the supFG1 gene (base pairs 167–176 and base pairs 167–196, respectively), contained within the SV40 vector, pSupFG1. The structure of the tethered psoralen (4′-hydroxymethyl-4,5,8-trimethylpsoralen) attached at the 4′-hydroxymethyl position via a two-carbon linker arm to the 5′-phosphate of the oligonucleotide is also shown. The psoralen-oligonucleotides are incubated with the SV40 vector DNA to allow site-specific triplex formation. Photoactivation of the psoralen by irradiation with either long wave ultraviolet light (UVA, 365 nm) or visible light (447 nm) is designed to generate adducts at the targeted intercalation site (166–167), as indicated by a black arrow. The oligomer-plasmid complex is then transfected into monkey COS-7 cells and allowed to replicate for 48 h. Following purification of the vector DNA by an alkaline lysis procedure, the DNA is used to transform E. coli SY204 lacZ125 (Am). Transformants are selected on ampicillin plates containing 5-bromo-4-chloro-3-indoly β-D-galactoside and isopropyl-β-D-thiogalactopyranoside for detection and isolation of mutants (white colonies) in which the supFG1 gene has been inactivated by mutation.

colonies were counted. The mutant colonies were purified, and the plasmids were isolated for DNA sequence analysis (13).
the supFG1 gene with equilibrium dissociation constants ($K_d$) of $8 \times 10^{-7}$ M and $3 \times 10^{-9}$ M, respectively (13).

The TFO and the vector DNA are coincubated in vitro to allow triplex formation, and either long wave UV light (UVA, centered at 365 nm) or visible light (447 nm) is used to photofreeze the psoralen to generate targeted photoadducts at base pairs 166–167 of the supFG1 gene. At the UVA dose of 1.8 $J/cm^2$ used in the present experiments, a previous analysis of the photoreactions on the context of the triple helix (using gel mobility shift assays and HPLC analysis) revealed that approximately 65% of the plasmid DNAs have a psoralen interstrand cross-link between the thymines in base pairs 166–167, with almost all of the cross-links oriented such that the furan-side adduct is on the T in base pair 166 and the pyrone-side adduct is on the T in base pair 167. In addition, 20% have a psoralen furan-side monooadduct at the T in base pair 166, 10% have a monooadduct (predominately pyrone-side) at the T in base pair 167, and 5% are not covalently modified (12). At a visible light dose of 1.17 $J/cm^2$, only 1% of the vectors have a psoralen interstrand cross-link between the Ts at 166–167, whereas 41% have a furan-side monooadduct on the T in base pair 166, 1% have a monooadduct on the T in base pair 167, and approximately 58% of the plasmids are not covalently modified (12). A monooadduct in the present discussion refers to the photodamaged of the tethered psoralen to just one strand of the duplex target, yielding a structure in which the TFO is covalently linked to that strand. A cross-link means that the psoralen has photoactivated with both strands of the duplex, yielding a structure in which the TFO is thereby covalently linked to both strands of the target DNA. A furan-side adduct refers to a lesion formed by cycloaddition to thymidine at the 4′-5′ bond of the furan ring of the psoralen, whereas a pyrone-side adduct refers to the product of cycloaddition involving the 3,4 bond of the pyrone ring. Although the previously published work focuses on photoadduct formation directed by pso-AG10, similar proportions of monooadduct formation have been observed with pso-AG30 (data not shown).

The plasmid-psoralen-oligonucleotide complex is transfected into monkey COS cells by electroporation. After 46 h for repair and/or replication, the vector DNA is isolated and used to transform $\lambda$ acZ (amber) bacteria for the genetic analysis of the supFG1 gene. Prior to the bacterial transformation, the DNA is subjected to digestion with DpnI, which restricts nonreplicated vector DNA that has not acquired the mammalian methylation pattern. This step eliminates unprocessed input molecules that might lead to misleading results.

Table I presents a comparison of the induced mutation frequencies in pSupFG1 following treatment with either pso-AG10 or pso-AG30 and either UVA or visible light. For both TFOs, UVA photoactivation of the tethered psoralen leads to more mutations than does visible light activation. This is not surprising, since the overall level of photoaduct formation is much higher with UVA. However, pso-AG10 appears to more efficiently generate mutations than does pso-AG30. This difference provided a preliminary indication that the third strand can affect the repair and mutagenesis of the damaged vector.

The mutants induced by pso-AG10 and pso-AG30 were analyzed by DNA sequencing (Fig. 2). With visible light activation, both pso-AG10 and pso-AG30 produced predominately T:A to A:T transversions at base pair 166. Since the main photoaduct following visible light irradiation is a furan-side psoralen monooadduct on the T in that base pair, these results are consistent with mutagenesis in both cases arising from translesion DNA synthesis across this adduct.

However, the mutations induced following UVA irradiation are distinct from those after visible light. Treatment of the vector with pso-AG10 and UVA generated mostly A:T to T:A transversions at base pair 167. This shift in mutation position correlates with the change in photoaduct distribution following UVA as opposed to visible light. After UVA, 75% of the plasmids have an adduct involving the T at 167, either a monooadduct (10%) or a cross-link (65%). Although the targeted cross-links consist of lesions at both 166 and 167, the data indicate that cross-link repair in this case leads predominately to mutations at 167.

In contrast, in the case of pso-AG30 plus UVA, A:T to T:A transversions are not seen at position 167. Some are found at 166, but the overall spectrum is, in general, more diverse, with mutations occurring over several base pairs around the psoralen intercalation site, including base pairs 165, 166, 167, and 168. In addition, although deletions around the target site are seen with both pso-AG10 and pso-AG30, larger deletions were seen with pso-AG30. These clear differences in the mutation spectra induced by pso-AG10 versus pso-AG30 suggest that repair of a triplex-targeted psoralen adduct can be influenced by the associated third strand.

Because the 30-mer binds more tightly to the target duplex than the 10-mer, and because it forms a triple helix that is longer than the standard nucleotide excision repair patch, we hypothesized that the pattern of mutations it induced was likely to reflect the effect of a third strand on repair to a much larger extent than in the case of the 10-mer. However, it remained a possibility that mutagenesis in the context of the 10-mer was also abnormal. Therefore, we designed an oligonucleotide that could deliver the psoralen to the target site at base pairs 166–167, but which could be detached from the adduct once it had been formed. To do this, an oligomer, pso-S-5-A9, was synthesized to contain a disulfide bond in the linker between the psoralen and the 5′ A in the AG10 sequence. Using this oligomer in conjunction with UVA photactiviation, targeted psoralen adducts were produced in pSupFG1. Release of the oligonucleotide fragment was achieved by treatment with dithiothreitol and heat, followed by size filtration to remove it from the sample and so prevent reformation of the triple helix. Using pso-S-5-A9 3′ end-labeled with $^{32}$P, successful detachment of the otherwise covalently attached oligomer was confirmed by gel electrophoresis and autoradiography of the vector DNA before and after oligonucleotide release (data not shown; complete details of this technique will be published elsewhere).

The vector DNA containing the site-specific adducts (but not the targeting oligonucleotide) was used to transfect COS cells as above, and the resulting mutations were analyzed. Out of 14 point mutations, 12 were found at 167, similar to those generated in the context of the 10-mer in the case of UVA photactiviation. Ten small deletions were also seen encompassing the target site. These results suggest that isolated psoralen adducts and adducts in the context of the 10-mer are handled by the cells in a qualitatively similar fashion. In contrast, pso-
AG30 adducts are processed in an altered way. In order to further elucidate potential differences in repair in the context of a triple helix, we examined repair of the plasmid-psoralen-oligonucleotide substrate in vitro in human cell extracts. We designed an assay to ask whether the triplex-targeted psoralen adduct is excised as predicted by the NER model of DNA damage repair. It has been shown in human cell extracts that dual incisions are made in the damaged DNA strand 6 nucleotides on the 3' side and 22 nucleotides on the 5' side of the damage (21). Helicase activity removes the damaged oligonucleotide fragment, and polymerase activity fills in the resulting gap (27). Based on this model, we hypothesized that excision repair of a triplex-targeted monoadduct would yield an oligonucleotide fragment containing a psoralen adduct tethered to the TFO, constituting a branched structure. Repair of a cross-link is envisioned to be more complicated, requiring sequential damage excision and repair synthesis on the two strands and/or interstrand recombination, as has been proposed (28–32). Hence, the expected products that may result from in vitro repair of a cross-linked substrate are less predictable.

To detect potential repair products, the TFOs were radioactively labeled at the 3' end using [α-32P]dideoxy-ATP and terminal transferase. The labeled TFOs, in conjunction with either UVA or visible light, were used to generate triplex-targeted psoralen adducts on pSupFG1. The psoralen-oligonucleotide-damaged plasmids were incubated in HeLa cell extracts under conditions as described for NER assays (25), and the products were analyzed by denaturing gel polyacrylamide electrophoresis and autoradiography (Figs. 3 and 4). Note that under the conditions of this gel analysis, neither triplex nor duplex interactions will persist; all noncovalent interactions will be disrupted, and the DNA fragments will be denatured. In Fig. 3, lanes 1 and 2 show as controls the input substrates formed by pso-AG10 and pso-AG30, respectively, incubated with pSupFG1 and irradiated with visible light. These samples include the plasmid covalently attached to the labeled psoralen-TFO (which is too large to enter the gel and is stuck at the top) and a residual amount of the labeled but unbound pso-TFO (which was not completely removed in the filtration step during sample preparation). Bands corresponding to pso-AG10 and pso-AG30 are therefore visible in these lanes (indicated by the arrows).

When the substrate from lane 1 (a pso-AG10-directed monoadduct) is added to the HeLa extract (lane 3), the extract generates a species of reduced mobility compared with the labeled pso-AG10 itself, consistent with a branched structure arising from excision from the plasmid of a damaged single strand fragment containing the psoralen-AG10 adduct. Much of the unbound, input pso-AG10 is degraded in the reactions and so is not visualized. In contrast to the case with pso-AG10, when a pso-AG30 and visible light-damaged plasmid is used as a substrate in the repair reaction (lane 4), no species of reduced mobility relative to pso-AG30 that might represent fragments released in repair are visualized.

When the substrate in the HeLa extract consists of pSupFG1 treated with pso-AG10 and UVA (Fig. 4, lane 2), a labeled fragment of reduced mobility relative to pso-AG10 alone (compare lane 1) is released from the plasmid. This band represents a fragment similar to that seen in Fig. 3, lane 3, in which the substrate was photoactivated by visible light. Since UVA treatment of pso-AG10/pSupFG1 generates both monoadducts and cross-links, whereas visible light produces only monoadducts, this species therefore likely arises from repair of the pso-AG10 monoadducts in the UVA-irradiated sample.

In the case of pso-AG30 and UVA-induced damage (Fig. 4, lanes 3 and 4), excision of a damaged DNA fragment appears to be inhibited. The position of the free pso-AG30 oligomer is shown in lane 4, and the results of incubation of the pso-AG30 and UVA-damaged plasmid in the HeLa extracts are shown in
Targeted psoralen adducts depending on the associated TFO (9, 13). In work in which the triplex-targeted adduct formation was carried out in vitro using pso-AG10 and UVA (9), we had previously observed mutations mostly at base pair 167. However, in experiments in which pso-AG30 was used to treat cells in culture (already containing the shuttle vector) and thereby to target psoralen adducts via in vivo triplex formation, we found mutations mostly at base pair 166 (13). Initially, we were concerned that there might be some difference in photochemistry inside versus outside of the cells that might alter the mutation distribution. However, the present study provides the explanation that the associated TFO itself, can influence psoralen adduct repair and mutagenesis.

A model to explain these results is illustrated in Fig. 5. In this diagram, using the psoralen adduct as a reference point, the potential endonuclease incisions in the NER pathway in each case are shown 6 nucleotides on the 3' side of the lesion and 22 nucleotides on the 5' side, as proposed for mammalian cells (21) (see Fig. 1 for the sequences and polarity of the strands in this diagram). Note that for both pso-AG10 and pso-AG30, the psoralen-TFO monoadducts occur mostly on the T in base pair 166 (the upper, purine-rich strand of the target duplex). We propose that excision of the pso-AG30 monoadducts (Fig. 5A, 3) is less efficient than of the pso-AG10 monoadducts (Fig. 5A, 1), because the longer triple helix overlaps the standard excision repair patch, potentially blocking the 5' incision. Also, the binding affinity of the 30-mer is 270-fold greater than that of the 10-mer, and so the 30-mer would be more likely to compete with and block NER proteins. The inhibition of repair endonuclease activity would be consistent with the inhibition of restriction enzymes by third strands that

DISCUSSION

We have examined psoralen mutagenesis targeted by triplex-forming oligonucleotides in the supFG1 gene carried in an SV40 shuttle vector passaged in COS cells. Although both pso-AG10 and pso-AG30 generated targeted mutations in the supFG1 following in vitro triplex formation and photoactivation, several differences were observed. Pso-AG10 generated higher mutation frequencies than did pso-AG30, especially following UVA irradiation. Also, the spectra of induced mutations following UVA irradiation differed between pso-AG10 and pso-AG30, with pso-AG10 generating mutations similar to those seen with isolated psoralen adducts at the same site. These results suggest that an associated triple helix can influence the repair and processing of targeted psoralen adducts, depending on the length and/or binding affinity of the third strand. In vitro repair experiments provide further evidence for the effect of the TFO on repair. These revealed an apparent inhibition of excision repair in the context of the 30-mer TFO in comparison with the 10-mer, as measured by release of a DNA fragment containing the psoralen-oligonucleotide adduct.

The results reported here confirm and extend our preliminary observations, suggesting differences in mutagenesis by

![Fig. 3. Excision repair of visible light-activated psoralen-oligonucleotide adducts in HeLa cell extracts.](image)

![Fig. 4. Excision repair of UVA-activated psoralen-oligonucleotide adducts in HeLa cell extracts.](image)
Repair of Triplex-targeted Adducts

A Repair

(1) psa-AG10 monoadduct

(2) psa-AG10 crosslink

(3) psa-AG30 monoadduct

(4) psa-AG30 crosslink

B Bypass replication

(1) psa-AG10 monoadduct

(2) psa-AG30 monoadduct

Fig. 5. Model for psoralen adduct repair (A) and bypass replication (B) in the context of a triple helix. The stick diagrams indicate the potential repair pathways for oligonucleotide-directed monoadducts and cross-links in the context of either psa-AG10 or psa-AG30, as indicated. (The sequences and the polarity of the strands in this diagram are presented in Fig. 1). The psoralen-conjugated oligonucleotides are represented by the smaller strands in each diagram, being connected to the duplex by either one line (monoadduct) or two lines (cross-link). The small arrows mark predicted sites of endonuclease incisions based on the reported properties of the nucleotide excision repair complex in mammalian cells, which generates incisions several base pairs (165–168) around the target site, and these are generated remains to be elucidated.

Since monoadduct repair should be relatively error-free, mutagenesis by the psa-TFO monoadducts, both in the case of psa-AG10 and psa-AG30, likely arises during bypass replication, as diagrammed in Fig. 5B. This model predicts that the induced mutation spectra for psa-AG10 and psa-AG30 monoadducts should be similar. As shown in Fig. 2, monoadducts targeted by the two psoralen-TFOs generated in both cases mostly T:A to A:T transversions at base pair 166, the proposed site of lesion bypass.

In the case of the UVA-induced cross-links, bypass replication would be blocked by the interstrand cross-link, and so much of the mutagenesis likely occurs during repair. Repair of psoralen cross-links is proposed to involve sequential excision repair, possibly coupled to recombinational pathways (28–32). In the case of free psoralen (without an associated TFO), the choice of the initial strand to be repaired is influenced by the orientation of the psoralen cross-link (furan-side lesion preferentially repaired in some systems (32)) and by the sequence context (33). For the psa-AG10 adduct (Fig. 5A, 2), repair may be biased toward the purine-rich strand (upper strand of the duplex in the diagram) for two reasons. First, the furan-side adduct in the cross-link is predominately on this strand (12). Second, the incisions on this strand would not be blocked by the triple helix, whereas the 3’ incision on the other strand (6 nucleotides from the psoralen lesion) would overlap with the triple helix region. Following excision repair of the damaged fragment from the upper strand, gap-filling repair synthesis would have to bypass the remaining lesion on the lower strand at position 167, leading to a mutation at that site. In accordance with this model, most of the mutations induced by psa-AG10 cross-links were A:T to T:A transversions at base pair 167, not at 166 as in the case of the monoadducts.

This model for cross-link repair and mutagenesis is further supported by the experiment in which targeted psoralen adducts were generated by psa-S–S-AG10 and UVA and then detached from the targeting oligonucleotide by disulfide bond reduction. The resulting vector substrate, containing predominately site-specific cross-links, yielded mostly A:T to T:A transversions at 167. This is consistent with cross-link repair involving excision of the damaged fragment from the upper strand, followed by gap-filling repair synthesis requiring bypass of the remaining lesion at 167.

An alternative pathway, in which the initial gap is filled via strand transfer from an undamaged template, is also possible and has been proposed (28–32). This would be followed by excision repair of the lower strand. In this case, an undamaged template for gap-filling synthesis would then be provided by the transferred strand. This scenario would therefore not require trans-lesion synthesis, and so it would be less likely to lead to mutations. This is consistent with the report of Sladek et al. (28) that psoralen cross-link-induced mutagenesis is suppressed in bacteria capable of recombination. Although this recombinational pathway may occur in mammalian cells, the pattern of targeted mutagenesis we have observed suggests that at least some of the cross-linked vectors are repaired in a mutagenic pathway that requires gap-filling repair synthesis across a damaged template.

With regard to psa-AG30-targeted cross-links, however, the potential sites of both the 5’ incision on the upper strand and the 3’ incision on the lower strand overlap the triple helix (Fig. 5A, 4). However, incision in the upper strand may be more strongly inhibited than in the lower strand, since the upper strand is the purine-rich strand to which the TFO directly binds by reverse Hoogsteen bonding in the antiparallel triple helix motif (3). If the incision in the lower strand is therefore less inhibited by the third strand oligonucleotide, then the second pathway illustrated in Fig. 5A, 4, would be favored. This pathway is predicted to involve bypass synthesis across a psoralen-damaged thymidine at position 166, and so it would be expected to lead to mutations at 166. As shown in Fig. 2, psa-AG30 plus UVA does lead to a plurality of T:A to A:T transversions at 166.

It is also possible, however, that the 166 mutations may not necessarily be a consequence of psa-AG30-directed cross-links. Instead, these mutations may arise, in part, from the 20% of the UVA-induced adducts that are monoadducts at 166. In addition, many of the mutations induced by psa-AG30 and UVA irradiation were found to occur not just at 166 but also at several base pairs (165–168) around the target site, and these are not specifically explained by the model. The mechanism by which these are generated remains to be elucidated.

If the usual cross-link repair pathway is, in fact, partially inhibited in the context of psa-AG30, we would expect that some mutations that normally arise in this pathway would be underrepresented in our assay. As shown in Fig. 2, no 167 A:T
to T:A transversions were observed. Also, the absence of a particular class of mutations might be expected to affect the overall mutation frequency. As shown in Table I, psoralen adducts produced fewer mutations than did psoralen adducts, it does not fully explain the mutation distribution induced by psoralen adducts and cross-links and by psoralen-directed monoadducts. Even a combination of TFOs can account for mutagenesis by psoralen adducts and cross-links. The results presented here, except for the association with cross-links, the genesis of the deletions, which are seen mostly in the case of the targeted monoadducts, suggest that excision of damaged DNA containing a psoralen adduct is at least partially blocked by the third strand. The exact mechanism by which NER is inhibited by the triple helix. Both the frequency and the spectrum of induced mutations were seen to vary depending on the length (and the binding affinity) of the TFO used to direct the psoralen adducts to the target site. In vitro experiments using HeLa cell extracts suggest that excision of damaged DNA containing a psoralen-TFO adduct is at least partially blocked by the third strand. The exact mechanism by which NER is inhibited by the triple helix, however, remains to be elucidated. Also, although the model proposed here can account for mutagenesis by psoralen adducts and cross-links and by psoralen-directed monoadducts, it does not fully explain the mutation distribution induced by psoralen-directed cross-links. Further work in this regard is required to examine the possible pathways of cross-link repair and mutagenesis in the context of an extended triple helix. Nonetheless, the results presented here, suggesting that triple helices can interfere with normal DNA repair pathways, bear on the use of TFOs for genetic manipulation. Furthermore, since triple helices can at least partially inhibit NER, TFOs may have utility as tools to probe DNA repair pathways.

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