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Repair of triplex-directed DNA alkylation by nucleotide excision repair

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ABSTRACT

Triplex-forming oligonucleotides (TFOs) are being investigated as highly specific DNA binding agents to inhibit the expression of clinically relevant genes. So far, they have been shown to inhibit transcription from the HER-2/neu gene *in vitro*, whereas their use *in vivo* has been studied to a limited extent. This study uses a TFO–chlorambucil (chl) conjugate capable of forming site-specific covalent guanine adducts within the HER-2/neu promoter. We demonstrate that nucleotide excision repair (NER) represents a mechanism of cellular resistance to TFO-directed DNA alkylation. *In vitro* repair assays demonstrate that triplex-directed chl–guanine adducts are substrates for repair by NER competent cell extracts but not XP12BE cell extracts deficient in NER. The degree of repair is estimated by a ligation-mediated polymerase chain reaction with a preformed triplex in a plasmid transfected into repair competent cells, indicating that ~25% of the guanine adducts are removed after 24 h. These data indicate that guanine adducts from TFO-directed alkylation are a substrate for NER and that DNA repair is a significant barrier to the intracellular persistence of target gene binding by TFOs.

INTRODUCTION

The field of 'anti-gene' therapy utilizing triplex-forming oligonucleotides (TFOs) as site-specific DNA binding agents has been of interest because of the ability of natural and modified oligonucleotides (ODNs) to recognize DNA sequences with high specificity. ODNs can be designed to bind double-stranded DNA targets through triple-helix formation by base-specific hydrogen bonds. Current studies are investigating the ability of these TFOs to modify gene expression as well as other DNA relevant biological processes. Triplex DNA formation has already been demonstrated on a number of clinically relevant genes, including c-*myc*, EGF-R, DHFR and HER-2/neu (1–5). The HER-2/neu oncogene appears to play an important role in the initiation and progression of many types of human cancer. Its overexpression has been observed with and without gene amplification in many human malignancies, including carcinomas of the breast (6) and lung (7–10). TFOs may help elucidate the role of HER-2/neu in cancer development and may also become a potential therapeutic tool.

Interstrand DNA triplex formation occurs by ODN binding in the major groove of a polypurine:polypyrimidine tract of DNA. Such tracts occur frequently in gene promoters, and the HER-2/neu gene contains two nearly homopurine tracts, one located in its promoter from –218 to –245 relative to the translation start site (Fig. 1) and the other in its coding sequence located from +31 to +54 (5). Targeting the promoter sequence can inhibit gene expression by competing with critical transcription factors required for transcription initiation. We have previously shown that triplex formation at the promoter target *in vitro* prevents protein binding to and transcription initiation from the HER-2/neu promoter (4), and other investigators have demonstrated that inhibition of transcription initiation is potentially due to the specific inhibition of protein binding by PU.1, a member of the *ets* family of transcription factors (11), and may reduce HER-2/neu expression in breast cancer cells (12). A second mechanism to potentially inhibit gene expression is inhibition of transcription elongation (13). We demonstrated that triplex-directed covalent modification of the target sequence on the template strand was necessary to prevent elongation by RNA polymerase through the HER-2/neu coding sequence (5), although others have shown that transcription elongation can be prevented with TFOs conjugated to intercalating agents (14) or TFOs with modified backbones that bind the DNA target with high affinity (15).

Demonstration of the ability of TFOs to inhibit target gene expression in living cells remains difficult. Barriers to intracellular triplex formation include factors that may prevent the TFO from reaching its target gene, such as nuclease degradation, self-association of guanine-rich ODNs, and chromatin structure. Other factors may prevent the formation of a triple helix even if the TFO reaches its target, such as the intracellular pH and ion composition (16). In addition, the triple helix, once formed, may be disrupted by DNA helicase (17). The helicase activity in RNA polymerase may account for the inability of TFOs to prevent transcription elongation in the HER-2/neu coding sequence unless triplex formation is coupled with covalent modification of the template strand (5). However, it is

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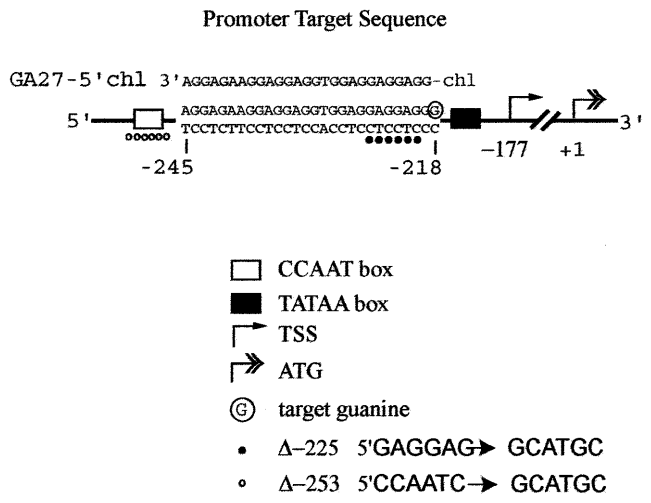

Figure 1. Schematic representation of the HER-2/neu gene. The numbering scheme is based on the translation initiation codon (ATG) as +1, with the primary transcription start site (TSS) at –177. The promoter triplex target sequence is located from –245 to –218 between the CCAAT (–249 to –253) and TATAA (–200 to –204) boxes. Mutant sequences are indicated. The TFO sequence and orientation relative to the target binding region and the location of chl conjugate is shown. The intended target guanine for alkylation is circled.

important to note that in spite of these barriers, there is now convincing evidence of intracellular TFO activity in yeast cells (18), in mammalian tissue culture cells (19) and in mice (20). In order to improve the stability of triplex formation and to prevent displacement of the TFO by helicase activity, we and others have used ODNs linked to molecules that will covalently modify the DNA target (5; reviewed in 16). For example, we have used DNA alkylating agents such as chlorambucil (chl), a bi-functional nitrogen mustard that reacts preferentially with the N7 of guanine bases (5). Although it is a bi-functional alkylating agent, when tethered to an ODN, mono-adduct formation at a guanine residue adjacent to the triple helix is much more common than cross-link formation (21). Guanine mono-adduct formation by an ODN–chl conjugate is illustrated in Figure 2.

The success of triplex-directed gene regulation is also dependent upon the efficiency and accuracy of cellular repair of the covalent DNA adducts. Intracellular repair of triplex-directed psoralen adducts in mammalian cells has been extensively studied, with some investigators reporting highly efficient repair (22,23) and others reporting inefficient repair (24,25). The discrepancies in these results may be attributed to various factors, including cell type, TFO length and orientation, and target gene studied. Recent repair studies in yeast and mammalian cells reported triplex-directed psoralen mono-adducts are efficiently repaired by transcription-coupled repair, nucleotide excision repair (NER) and error-prone repair pathways, while interstrand cross-links are poorly repaired (19,26,27). The repair of chl and other chemical moieties being used in conjunction with TFOs has not been extensively studied. We hypothesized that NER may be an important pathway in the repair of chl-induced DNA damage, since NER is known to repair bulky lesions that modify DNA structure, and has been shown to be involved in the repair of synthetic apurinic (AP) sites and cisplatin-induced intrastrand crosslinks (28,29).

In this paper, we have used triple-helix formation by a TFO–chl conjugate in the HER-2/neu promoter to characterize the repair of triplex-directed covalent guanine adducts by the NER pathway. After demonstrating site-specific guanine alkylation by the TFO–chl conjugate, we used *in vitro* repair assays to demonstrate that the guanine adducts formed were substrates for NER. Using a method based on piperidine cleavage and ligation-mediated polymerase chain reaction (LM-PCR), we detected triplex-directed guanine alkylation of the HER-2/neu gene in a plasmid after transfection into repair competent cells and estimated the extent of guanine adduct formation that remained after transfection. The future therapeutic potential for TFOs used to direct site-specific DNA adducts will depend upon the development of novel repair-resistant and intracellularly stable TFO–alkylator conjugates.

MATERIALS AND METHODS

Oligonucleotide synthesis

ODNs from commercial sources were purified by denaturing polyacrylamide gel electrophoresis. The ODN–chl conjugate was synthesized by Epoch Pharmaceuticals (Bothell, WA) as previously described (30). Conjugates were stored at –80°C and thawed on ice prior to use. The sequence of the HER-2/neu promoter and the TFO is given in Figure 1, and the structure of the ODN–chl conjugate is illustrated in Figure 2.

Plasmid constructs

The construct pGL3/HNP410 contains 410 bp of the HER-2/neu promoter from base –410 to +1 relative to the translation start site ligated into the *Xho*I and *Nco*I sites of pGL3 basic (Promega), and maintains the relative location of the HER-2/neu promoter triplex target sequence at –245 to –218. Site-directed mutagenesis was performed on this plasmid using an established protocol (Quickchange, Stratagene) to generate mutant plasmids. An *Sph*I restriction site was engineered upstream of the triplex target sequence at –253 to –248 [pGL3/HNP410(Δ-253)] and within the triplex target sequence at –225 to –220 [pGL3/HNP410(Δ-225)]. The Δ-253 mutant (CCAATC→GCATGC) introduced a convenient restriction enzyme site into the HER-2/neu promoter for use in the LM-PCR assay; the Δ-225 mutant (GAGGAG→GCATGC) contains a 6 bp mutation that disrupts triplex formation at the downstream end of the triplex target and thus inhibits alkylation of the TFO–chl conjugate.

Piperidine cleavage assay

Piperidine cleavage and the standard Maxam–Gilbert G-reaction were performed as described previously (31). A 394 bp *Pst*I/*Nco*I fragment of the pGL3/HNP410 plasmid was labeled by the Klenow fill-in reaction. Triplex formation was performed by incubating 25 ng (~100 fmol) of labeled promoter fragment with increasing concentrations of the TFO–chl conjugate in 1× TBM (90 mM Tris pH 8, 90 mM borate and 10 mM $MgCl_2$) in a final volume of 10 µl. Triplex mixtures were incubated at 37°C for the times indicated. These mixtures were then added to 1 M piperidine at a final volume of 100 µl, heated at 80°C

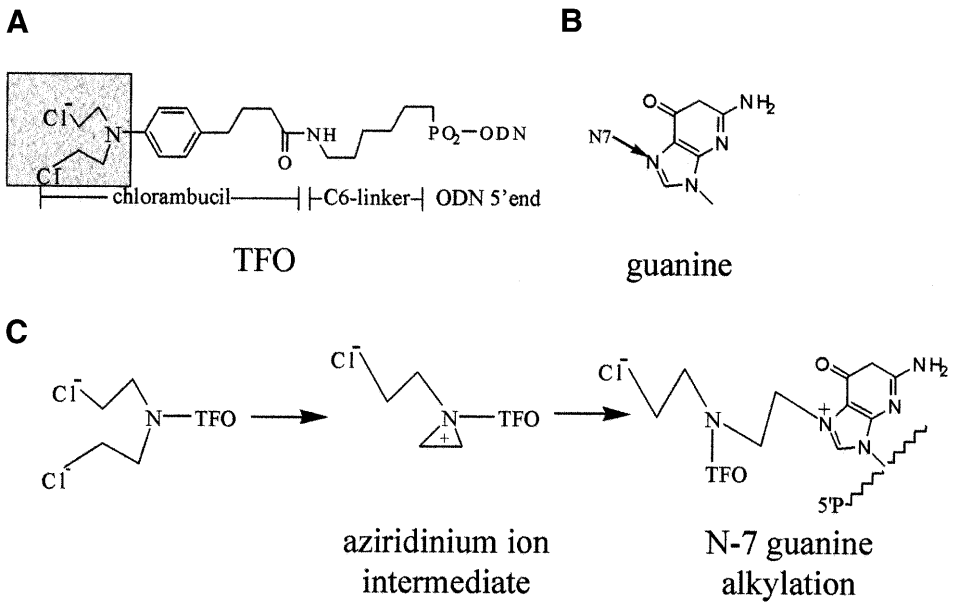

Figure 2. (**A**) Schematic representation of the ODN–chl conjugate, showing the amide bond formed by condensing a terminal C6-amino group appended to the ODN with the carboxyl group of the chl. The reactive chloroethylamine groups of chl, a motif common to the nitrogen mustards, are highlighted. (**B**) Structure of guanine with the N-7 position indicated by an arrow. (**C**) Schematic representation of the molecular events involved in the formation of a guanine adduct by chl conjugated to an oligonucleotide (TFO). The chloroethylamine arm undergoes intramolecular rearrangement to form a highly reactive aziridinium ion intermediate that attacks the N-7 of guanine.

for 20 min and lyophilized extensively before electrophoresis on an 8% denaturing polyacrylamide sequencing gel.

In vitro repair assay

Plasmids containing intact triplex target (pGL3/HNP410) or mutated target [pGL3/HNP410(Δ-225)] were incubated at 37°C overnight with the TFO–chl conjugate in 1× TBM. Following triplex formation with the TFO–chl conjugate, plasmids were treated with 100 µg HeLa or XP12BE cell-free extracts for 3 h in the presence of dNTPs and ^{32}P-dATP as described (32). Cell-free extracts were prepared as previously described (33) but with an increased time of incubation in isolation buffer to allow elution of nuclear proteins. Plasmids were then digested with *Eco*RI and *Nco*I to excise the HER-2/neu promoter fragment containing the triplex target site and isolated on a 1% agarose gel. Incorporation of ^{32}P-dATP in the fragment containing the triplex target site indicates repair of guanine adducts formed by triplex-directed DNA alkylation.

Ligation-mediated polymerase chain reaction on transfected plasmids

To estimate the degree of *in vivo* DNA repair, plasmid DNA containing a triple helix formed by the TFO–chl conjugate was transfected into HeLa cells. The degree of repair was estimated by the persistence of the mono-adduct as detected by LM-PCR. Triplex formation was performed with the supercoiled plasmid [pGL3/HNP410(Δ-253)] under conditions optimized for triplex formation and similar to those described for the piperidine cleavage assays. Unbound TFO–chl conjugate was removed by passage over a 1× TBM equilibrated Chromaspin-100 column (Clontech). An aliquot of 2 µg of the plasmid containing the triple helix was then added to *Trans*IT-LT1 (Mirus, Madison, WI) as per the manufacturer's instructions, and the transfection mixture was added to HeLa cells growing in log phase. After 24 h, the cells were washed three times in phosphate-buffered saline and the soluble nucleic acid fraction was isolated using a commercially available total RNA isolation kit (Qiagen). The concentration of soluble nucleic acid was determined by spectrophotometry in an attempt to equalize the amount of plasmid used in subsequent reactions, and the plasmid was isolated from the total RNA by DNase free RNase A digestion and ethanol precipitation. The plasmid contained in 10 µg of the soluble nucleic acid fraction was then subjected to complete digestion with *Sph*I followed by piperidine cleavage. This DNA was then used as a substrate in LM-PCR. HER-2/neu promoter-specific primers used for LM-PCR are as follows: primer 1, 5′-GGTTTCTCCGGTCCCAA; primer 2, 5′-TCCCAATGGAGGGGAATCTCA; primer 3, 5′-CAATGGAGGGGAATCTCAGCTTCAC. The asymmetric linker was produced by annealing 21mer (5′-TGACACGGGAGATCTGAATTG) and 11mer (5′-CAATTCAGATC) ODNs in the presence of 250 mM Tris pH 7.7. LM-PCR was performed as described previously (34) with minor modifications. First-strand synthesis was performed by Sequenase T7 DNA polymerase (USB) using primer 1. AmpliTaq DNA polymerase (Perkin Elmer) was used for PCR amplification using primer 2 and the 21mer primer. The labeling reaction used 10 pmol of end-labeled primer 3 per reaction, with heating to 95°C for 2 min, then 76°C extension for 12 min. These reactions were then resolved on a denaturing 8% polyacrylamide gel followed by autoradiography. Extension products are expected to yield bands of defined length representing cleavage at either the *Sph*I site or at the site of the guanine adduct at –218.

RESULTS

The initial goal of our studies with TFO–chl conjugates was to demonstrate that triplex formation in the HER-2/neu promoter could prevent transcription initiation in cells. Transient transfection experiments were designed to determine whether preformed triplex formation with TFO–chl conjugates could inhibit HER-2/neu transcription initiation from a plasmid transfected into various human cancer cell lines. The results of these studies were inconsistent and frequently negative (data not shown), leading us to hypothesize that intracellular repair mechanisms may be responsible for the lack of consistent transcriptional inhibition by a preformed triple helix in transiently transfected plasmids.

The triplex target sequence of the HER-2/neu promoter is illustrated in Figure 1. The promoter target is located from –218 to –245 relative to the translation start site (+1). Triplex formation by purine-rich ODNs at this site has been previously shown to prevent *in vitro* transcription initiation (4). The TFO illustrated in Figure 1 is purine rich and binds in antiparallel orientation to the purine strand of the triplex target sequence.

Site-specific guanine alkylation by GA27–5′chl

The ODN–chl conjugate (GA27–5′chl) was designed to overlap 27 nt of the promoter target sequence and alkylate guanine –218 located on the purine-rich strand adjacent to the triple helix. Figure 2 illustrates the structure of the ODN–chl conjugate and the formation of a guanine adduct with the N7 position of guanine. Chl undergoes spontaneous rearrangement in aqueous solution to form a highly reactive aziridinium ion intermediate. Chl has a half-life of ~30 min in aqueous solutions, and the reactivity of ODN–nitrogen mustard conjugates is affected by the concentration of chloride ions and the presence of nucleophiles (30). Sites of DNA alkylation by the chl moiety can be identified by piperidine cleavage.

Figure 3 demonstrates a piperidine cleavage reaction after increasing the concentration of the TFO and the time of incubation with the radiolabeled promoter target (*Pst*I/*Nco*I fragment). Lane 1 is a G-reaction, which enables identification of the alkylated G-residue. Target guanine alkylation at G-218 represents ~50% of the cleavage products with lesser reactions occurring with adjacent guanine bases –219 and –220 (each represents ~25% of the cleavage products), and there is no evidence of alkylation at guanine bases outside the triplex target. Alkylation efficiency, estimated by the band intensity of the cleavage products compared to the full length radiolabeled promoter fragment, exceeds 80% with GA27–5′chl after a 2 h incubation, but requires a high concentration (20 μM) of TFO to achieve this high level of alkylation. Triplex formation with this target sequence can occur at nanomolar concentrations of TFO (4), but much higher concentrations of the chl-conjugated TFO are required for efficient guanine adduct formation. Because triplex formation precedes and directs guanine alkylation, the large molar excess of the TFO fosters rapid binding of the TFO to the DNA target (present at very low concentrations), is necessary for efficient alkylation due to the short half-life of chl in aqueous solution, and may be improved with reactive agents that have longer half-lives (30). Piperidine cleavage analysis of the complementary strand shows very inefficient alkylation of G-217. This finding indicates that triplex formation

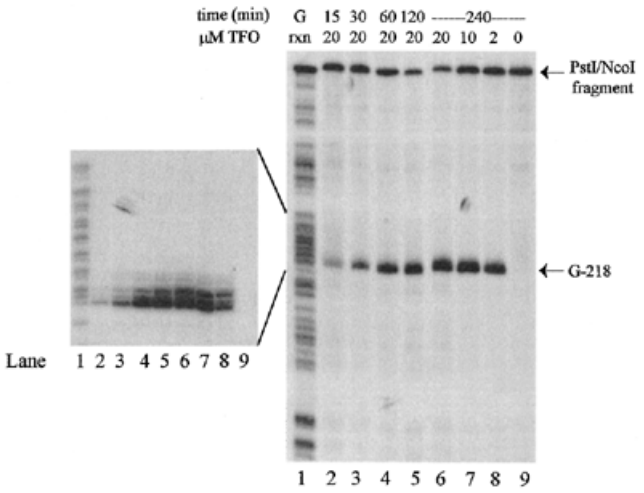

Figure 3. Site-specific alkylation of G-218 in the HER-2/neu promoter is demonstrated by piperidine cleavage analysis. The Maxam and Gilbert G-reaction of the HER-2/neu promoter template (lane 1) is used to identify the location of the triplex target sequence and piperidine cleavage sites. Lanes 2–9 are labeled promoter *Pst*I/*Nco*I fragments incubated with the indicated concentration of GA27–5′chl for increasing amounts of time. Finer detail in the inset demonstrates that adducts are formed predominantly with guanine –218 adjacent to the triple helix, with lesser amounts of adduct formed with neighboring guanines –219 and –220 within the triple helix.

with TFO–chl conjugates generally leads to mono-adduct formation rather than interstrand crosslink formation even when an appropriate target for interstrand crosslink formation is present (data not shown). Site-specific guanine alkylation has also been previously demonstrated at the HER-2/neu coding target using GA23–5′chl, also with no detectable alkylation at guanines not adjacent to the triple helix (5). These data support the conclusion of Lampe and co-workers that the chl moiety in an ODN–chl conjugate does not react on its own but only when the initial binding to DNA is mediated by the TFO (21).

In vitro repair of guanine adducts requires nucleotide excision repair

We investigated the possibility that the NER pathway is responsible for the repair of triplex-directed chl adducts. For these experiments, we performed *in vitro* repair assays with cell-free extracts from repair competent HeLa cells or NER deficient XP12BE cells (immortalized fibroblasts from a patient with xeroderma pigmentosa, complementation group A, lacking a specific protein from the excision nuclease complex) as shown in Figure 4. Triplex formation with GA27–5′chl (+TFO) occurred on supercoiled plasmids containing either native (pGL3/HNP410) or mutant [pGL3/HNP410(Δ-225)] HER-2/neu promoter. This mutation from bases –225 to –220 disrupts triplex formation at the distal end of the triplex target and prevents subsequent guanine alkylation at the triplex target by the TFO–chl conjugate. After triplex formation, the plasmid was incubated with cell-free extracts in the presence of dNTPs and ^{32}P-dATP so that the repaired patch of DNA will contain the radiolabeled ^{32}P-dATP. The plasmid was then digested with *Eco*RI and *Nco*I to excise a 394 bp fragment of the HER-2/neu promoter.

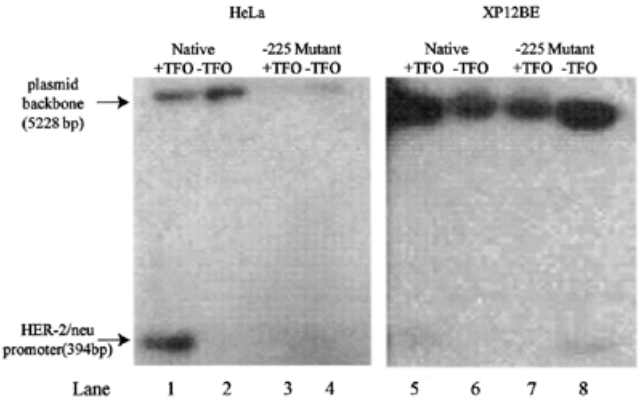

Figure 4. *In vitro* repair assay with HeLa cell extracts (repair competent) or XP12BE cell extracts (NER deficient). Repair is detected by the incorporation of ^{32}P-dATP specifically into the restriction fragment (394 bp) of pGL3/HNP410 containing the HER-2/neu promoter region with the guanine adduct due to triplex-directed alkylation by GA27–5′chl (+TFO). The mutant plasmid (−225) is capable of triplex formation with only part of the TFO and is unable to undergo alkylation at the target guanine residue, and serves as a negative control. No specific repair of the guanine adduct is detected in the native plasmid treated with or without TFO by the XP12BE extracts.

The specific incorporation of ^{32}P-dATP is seen only in the 394 bp restriction fragment in lane 1 of Figure 4, which represents native plasmid treated with GA27–5′chl, and then incubated with HeLa cell-free extracts. In the absence of TFO–chl (–TFO, lane 2), the native plasmid shows only a small amount of background dNTP incorporation into the plasmid backbone (probably due to repair of plasmids that are randomly damaged during preparation or freeze/thaws). Random DNA damage will result in the incorporation of dNTPs so that band intensity is proportionate to the size of the DNA fragment. The restriction fragment containing the bound TFO–chl is only one-fifteenth the size of the plasmid backbone. The 4-fold increase in band intensity in the restriction fragment containing the TFO–chl adduct compared to the plasmid backbone in lane 1 indicates that repair is specific to the restriction fragment containing the guanine adduct. The mutant plasmid shows only trace amounts of background dNTP incorporation into the plasmid backbone in the presence or absence of TFO–chl (lanes 3 and 4); there is less background incorporation of dNTPs in the mutant plasmid that likely resulted from a lower amount of random DNA damage during handling in this particular plasmid's preparation. An overexposure of this autoradiograph (not shown) indicates radiolabel incorporation in direct proportion to the size of the plasmid backbone and *Eco*RI/*Nco*I fragments in the pGL3/HNP410(Δ-225) plasmid due to random damage. Overexposure of the autoradiograph from the XP12BE extract-treated plasmids shows only backbone dNTP incorporation in both plasmids in the presence or absence of triplex-directed DNA alkylation (lanes 5–8). This background incorporation may be due to repair of random DNA damage by other functional repair pathways, such as base excision repair (BER).

Semi-quantitative detection of triplex persistence *in vitro* and post-transfection

In order to estimate the extent of repair of triplex-directed DNA alkylation in HeLa cells, we utilized the LM-PCR. LM-PCR has been developed for footprinting studies of protein:DNA interactions in genomic DNA samples, especially for *in vivo* footprinting in living cells. LM-PCR is based on the principle of detecting the cleavage products of a footprinting reagent (such as dimethyl sulfate followed by piperidine cleavage) in a gene of interest within a genomic DNA sample by primer extension with a gene-specific primer followed by the blunt ligation of an asymmetric linker (designed to ligate in only one orientation) that is not gene specific. PCR amplification with a nested, gene-specific PCR primer and a primer corresponding to the sequence of the linker yields detectable levels of the family of cleavage products that can then be resolved by gel electrophoresis and detected by autoradiography after a final nested primer extension with an end-labeled primer (34). The technique of LM-PCR has been used to detect triplex-directed covalent adduct formation with a single guanine base in genomic DNA (35). Because the triplex-directed guanine adduct can be converted to a single strand break after piperidine cleavage, LM-PCR can identify the unique cleavage product at this site in genomic DNA. Furthermore, the extent of genomic targeting by the TFO alkylator was estimated by competitive LM-PCR after complete digestion of the genomic DNA at a known restriction site upstream of the triplex target (35).

We used LM-PCR in this study to evaluate the amount of the triplex-directed guanine adduct that persisted in the HER-2/neu promoter reporter plasmid after its transfection into repair-competent HeLa cells. In this assay, site-specific guanine adducts are quantified by using a HER-2/neu promoter reporter plasmid, pHNP410(Δ-253), with an *Sph*I site engineered upstream of the triplex target sequence. *Sph*I cleavage of the HER-2/neu promoter at –253 upstream of the triplex target yields 100% cleavage, and this cleavage site serves as a competitor for LM-PCR quantitation for triplex-directed adduct formation at the downstream guanine target. After *Sph*I digestion and piperidine cleavage, LM-PCR will yield two bands on a sequencing gel representing the –218 and –253 cleavage sites in the HER-2/neu promoter, illustrated schematically in Figure 5. Triplex-directed alkylation efficiency and the persistence of the guanine adduct at G-218 is determined by the ratio of band intensity at –218 to the sum of band intensities at –218 plus –253. A standard curve was constructed by mixing known ratios of untreated plasmid with plasmid after treatment with the TFO–chl (lanes 1–5). Since this is a PCR-based method, the overall band intensities can be altered by minor differences in the amount of starting DNA template, but the relative band intensities at the –218 and –253 cleavage sites are preserved as a result of the competitive PCR reaction.

pHNP410(Δ-253) was either 100% bound by the TFO–chl conjugate (+TFO) or untreated (–TFO), and the reactions were split for transfection (Fig. 5, lanes 8 and 9) or for a control LM-PCR reaction (lanes 6 and 7). Twenty-four hours after transfection into HeLa cells, the plasmid DNA was extracted and then subjected to *Sph*I digestion, piperidine cleavage and LM-PCR. Comparing treated and untreated plasmid pre- and post-transfection (lanes 6–9), it is clear that the amount of alkylation at G-218 decreases significantly post-transfection, as noted by the appearance of the band representing the *Sph*I site at –253 (lane 9). By comparison with the LM-PCR controls, we estimate that ~25% of the guanine adduct is removed within 24 h by HeLa cells. These data suggest that HeLa

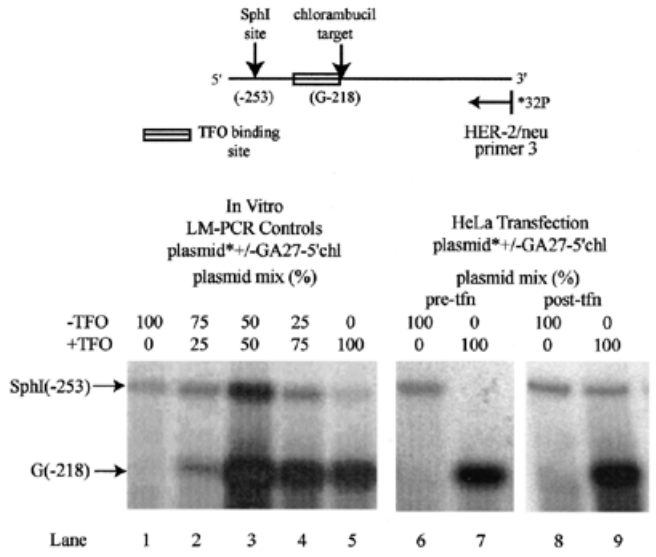

Figure 5. LM-PCR assay demonstrating the limited persistence of triplex-directed guanine mono-adducts in repair-competent HeLa cells. The upper schematic indicates the location of the control *Sph*I cleavage site and target alkylation site relative to LM-PCR primer 3. Lanes 1–5 show LM-PCR controls performed *in vitro* with increasing amounts of triplexed plasmid (+TFO) to unmodified plasmid (−TFO) to provide a standard curve. HeLa cells were transfected with pGL3/HNP410(Δ-253)(plasmid*) treated with GA27−5′chl (+TFO) or without TFO (−TFO). Lanes 8 and 9 represent plasmid transfection into HeLa cells while lanes 6 and 7 represent the same triplex reactions incubated *in vitro*.

cells, competent in NER, are capable of repairing a significant number of triplex-directed guanine adducts (25%) within a relatively short time (24 h).

DISCUSSION

Our studies have shown that NER is a significant barrier to the strategy of gene targeting with TFOs conjugated to chemically reactive agents such as nitrogen mustards. We believe that our studies are the first to characterize the pathway of DNA repair of this class of TFO conjugates as well as the first to attempt to assess the extent of persistence of TFO-directed guanine adducts in cells. It is not surprising that NER is involved in the repair of triplex-directed guanine adducts, since the guanine adducts formed by other bulky DNA alkylating agents are also repaired by NER (29). These data do not specifically address the involvement of other pathways in the repair of guanine adducts that result from TFO-directed alkylation, such as base excision repair (BER). One concern is that depurination at the guanine adduct sites will lead to BER rather than NER of the resulting AP site. For example, depurination at 5–10% of guanine adduct sites was observed in one study of triplex-directed DNA alkylation with the bifunctional alkylating agent diazyridinylquinone (36), while another study demonstrated that depurination was not observed *in vitro* using triplex-directed DNA alkylation with chl (37). In our studies, we believe that BER is an unlikely explanation for the repair of triplex-directed TFO–chl guanine mono-adducts because XP12BE cells are capable of effective BER, and in our *in vitro* repair assay, XP12BE extracts were incapable of repairing the TFO-directed guanine adducts at detectable levels.

The success of antigene ODNs designed to form a targeted covalent DNA adduct is highly dependent upon the efficiency of recognition and repair by intracellular mechanisms. In our studies, a significant proportion (25%) of the triplex-directed guanine adducts are repaired within repair competent cells in 24 h, demonstrating that DNA repair is an important barrier to the successful application of antigene oligonucleotides *in vivo*. In this regard, TFO–psoralen conjugates and the mechanisms for DNA damage repair of their UV photoproducts have been well studied (19,22,23,25,27,38–41). One report using TFO–psoralen conjugates suggests that repair of a single site of DNA damage, even of a psoralen interstrand crosslink, is efficiently repaired in mammalian cells, while crosslinks at both ends of the triple helix are inefficiently repaired (25). This observation may extend to TFOs conjugated to nitrogen mustards, and a bis-conjugated TFO that forms adducts at both ends of the triple helix may be able to resist NER. Future studies are needed to develop TFO conjugates capable of resisting DNA damage repair.

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