Triple helix-forming oligonucleotides target psoralen adducts to specific chromosomal sequences in human cells

Dennis H. Oh* and Philip C. Hanawalt

Department of Biological Sciences and Department of Dermatology, Stanford University, Stanford, CA 94305-5020, USA

Received August 30, 1999; Revised and Accepted October 27, 1999

ABSTRACT

The ability to target photochemical adducts to specific genomic DNA sequences in cells is useful for studying DNA repair and mutagenesis in intact cells, and also as a potential mode of gene-specific therapy. Triple helix-forming DNA oligonucleotides linked to psoralen (psoTFOs) were designed to deliver UVA-induced psoralen photoadducts to two distinct sequences within the human interstitial collagenase gene. A primer extension assay demonstrated that the appropriate psoTFO selectively damages a collagenase cDNA target. Site-specific genomic psoTFO DNA adducts were detected by a single-strand ligation PCR assay. The adduct, formed at a single site by a psoTFO in purified genomic DNA, contrasted with the multiple sites that were damaged within the observed segment of the collagenase gene upon treatment with free psoralen and subsequent photoactivation. When treated with psoTFOs, both repair-deficient fibroblasts from xeroderma pigmentosum complementation group A and HT1080 fibrosarcoma cells exhibited site-specific DNA adducts following UVA irradiation. Addition of phorbol ester, a transcriptional activator of the collagenase gene, to xeroderma pigmentosum cells did not detectably alter the initial levels of damage produced by psoTFOs, suggesting that further stimulation of transcription neither improves accessibility of psoTFOs to their targets nor enhances removal of non-covalently bound psoTFOs.

INTRODUCTION

Generation of sequence-specific and strand-specific psoralen photoadducts in the genome of living mammalian cells is an intriguing strategy to study in detail the effects of DNA damage induced by psoralens and to explore gene-specific therapies. Recently, triple helix-forming oligonucleotides (TFOs) have been shown to be promising reagents for specifically binding to the major groove of purine-rich sequences and delivering different chemically reactive moieties to DNA (1–3). Most work has described TFO-induced modifications of exogenous plasmid vectors that are treated either before or following introduction into cells. Chromosomal mutations resulting from TFOs have recently been reported in mammalian cells (4,5). Equally challenging and important has been the direct demonstration of TFO binding to native chromosomal DNA inside living cells, which has thus far required physical permeabilization of cells to facilitate sufficient entry of the TFOs (6,7). Potentially complicating interpretation of previous intracellular binding experiments, however, TFOs that are not specifically bound following treatment of living cells can in some circumstances persist in subsequent steps of a detection assay and produce binding artifacts unless they are explicitly removed (8).

We describe here the use of single-strand ligation PCR (sslig-PCR) to demonstrate unambiguously that TFOs covalently linked to psoralen (psoTFOs) bind and damage specific native chromosomal DNA target sequences, both in cell-free systems and in intact human fibroblast-derived cells grown in monolayer cultures. The target chosen, the human interstitial collagenase gene, is a key member of the matrix metalloproteinase family that degrades connective tissue and that is over-expressed in a variety of inflammatory and degenerative diseases (9). In most cases, overexpression is mediated by increased transcriptional activity and, while several inhibitors of collagenase activity are known, none specifically inhibits the transcriptional activity of a single gene. Our results with TFOs indicate that they are effective tools for introducing site-specific lesions into the genomes of living cells, and that they may be useful for studying DNA repair as well as for eventually modulating the activity of specific target genes.

MATERIALS AND METHODS

Chemicals

The psoralen derivative 4′-hydroxymethyl-3,4,5′-trimethylpsoralen (HMT) was obtained from HRI Associates (Concord, CA). 12-0-Tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma (St Louis, MO).

Oligodeoxyribonucleotides

DNA oligonucleotides were either gel or HPLC purified (Oligos Etc., Wilsonville, OR). The psoTFOs used are shown in Figure 1. Each TFO had HMT covalently linked by either a two- or six-carbon linker to the 5′-terminus of the oligonucleotide.

*To whom correspondence should be addressed at: Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020, USA. Tel: +1 650 723 2425; Fax: +1 650 725 1848; Email: doh@leland.stanford.edu
For epifluorescence microscopy and flow cytometry, the same psoTFOs also possessed fluorescein isothiocyanate (FITC) at their 3′-termini. Each TFO possessed a phosphodiester backbone, except for phosphorothioate linkages for the last two residues at the 3′-terminus. In some cases, oligonucleotides used as primers in the assays described below were 5′-end-labeled with [γ-32P]ATP (10).

Treatment of cDNA or genomic DNA with psoTFOs

Human collagenase cDNA in a plasmid, pBScoll.dna, was provided by W. Hoeffler. Prior to use, the plasmid was linearized with PstI, resulting in loss of the terminal quarter of the cDNA sequence, but preserving the psoTFO1 binding site in the remaining cDNA fragment (11). Genomic DNA was prepared from HT1080 cells as previously described (12). DNA was then incubated with the appropriate psoTFO in a reaction buffer consisting of 10 mM Tris, pH 7.5, 20 mM MgCl2, and 1 mM spermidine at room temperature for 1–2 h prior to irradiation.

Cells

HT1080 fibrosarcoma cells were obtained from the American Type Culture Collection. XP12RO-SV40 is an SV40-transfected xeroderma pigmentosum group A cell line (13). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C, 5% CO2.

Transfection

One day before transfection, cells were plated at a density of 104/cm2 in DMEM supplemented with 10% FBS. One hour prior to transfection, the medium was replaced with serum-free DMEM. psoTFOs were transfected into cells using LipofectAMINE PLUS according to the manufacturer’s guidelines (Life Technologies, Gaithersburg, MD), resulting in a final psoTFO concentration of 250 nM. psoTFO concentrations >250 nM in the presence of LipofectAMINE PLUS resulted in precipitate. Cultures were incubated for 2 h at 37°C, then at room temperature for 30 min, after which they were either irradiated or visualized by microscopy.

Irradiation

UVA exposures were measured with an IL1400A photometer equipped with an SEL033 UVA detector (International Light Inc., Newburyport, MA). The UVA source was a flat array of 15 W BL fluorescent lamps (UVP, Upland, CA) that produced light predominantly between 300 and 400 nm, with 98.4% of the irradiance between 320 and 400 nm. A 4.5 mm plate glass filter between the lamps and samples ensured that the irradiance below 320 nm was reduced by at least an additional 97%. In cell-free experiments, samples in polypropylene tubes were irradiated from above with an irradiance of 1.7 mW/cm². For cellular experiments, monolayer cell cultures were washed in phosphate-buffered saline (PBS), then irradiated with 2.5 mW/cm² through the bottom of the culture dishes in a cold room at 4°C. Experiments requiring wavelengths >395 nm employed a 100 W Hg arc lamp (Universal Light Source, San Francisco, CA) in a custom built housing. The broadband lamp output was diffused through frosted glass, and filtered through a GG400 glass filter (Schott Glass Technologies, Duryea, PA), and 1.4 cm path length filters made with aqueous solutions of CuSO4 (250 g/l) and CoSO4 (240 g/l) (14). Broadband light (390–450 nm) centered at 410 nm resulted, with an irradiance of 11 mW/cm² at the beam focus, measured by a LI-185B quantum photometer equipped with a LI-190SB sensor (LI-COR, Lincoln, NB); wavelengths below 400 nm accounted for <0.25 μW/cm². The sample was continuously mixed with a magnetic stir bar in a cuvette placed at the beam focus.

 Primer extension assay

Linearized pBScoll.dna plasmid (95 ng), treated with psoTFOs as described above, was incubated with 1 U Taq polymerase in 20 mM Tris, pH 8.4, 50 mM KCl, 200 μM each dNTP, 1.5 mM MgCl2, and 10 nM of a 3′-5′-end-labeled primer, d(ATGACTTTCTGGAAATTGGCC), that bound 69 and 145 bp upstream of the psoTFO1 binding site and the PstI restriction site, respectively (22). A GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT) denatured the reaction mixture at 94°C for 5 min, then performed 25 cycles of 94°C for 2 min, 55°C for 55 s, and 72°C for 2 min followed by completion of the reaction at 72°C for 10 min, and cooling to 4°C. Products were precipitated with ethanol, separated by denaturing polyacrylamide gel electrophoresis, and analyzed by autoradiography and phosphorimaging.

Single-strand ligation PCR

Psoralen adducts in defined sequences of genomic DNA were assayed by adapting a method previously described for analyzing cisplatin adducts, as outlined in Figure 2 (15,16). DNA was prepared from cells as previously described (12), and digested with an appropriate restriction endonuclease to generate a DNA fragment with a free end downstream of the intended psoTFO target site. The DNA was then amplified in the region of interest by allowing Taq polymerase to extend a biotinylated primer (primer 1) binding upstream of the expected lesion, generating either a ‘full-length’ DNA product whose 3′-end was defined by the restriction endonuclease site and that corresponded to undamaged DNA, or a truncated product whose 3′-end was defined by any intervening DNA lesions capable of blocking the polymerase. Primer extension (linear PCR) was performed on 1–3 μg of genomic DNA in a volume of 40 μl, in the presence of 20 mM Tris, pH 8.4, 50 mM KCl, 200 μM each dNTP, 1.5 mM MgCl2, 15 nM biotinylated primer 1, and 1 U Taq DNA polymerase (Life Technologies, Gaithersburg, MD). The reaction mixture was denatured at 94°C for 5 min, and then subjected to 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min initially, with a 1 s extension for each subsequent cycle, followed by termination at 72°C for 5 min and cooling to 4°C. The biotinylated primer extension products were captured on streptavidin-coated magnetic beads (M-280 Streptavidin Dynabeads; Dynal, Oslo, Norway) by incubation at 37°C for 30 min in 5 mM Tris, pH 7.6, 1 mM EDTA, 1 M NaCl. The captured products were isolated on a magnetic rack, washed thrice in TE buffer (10 mM Tris, pH 7.6, 1 mM EDTA), and their 3′-ends were ligated to the 5′-end of a ligation oligonucleotide by incubating the magnetic beads in a mixture consisting of 25% PEG 8000 (Sigma, St Louis, MO), 20 μM ligation oligonucleotide, 50 mM Tris, pH 7.8, 10 mM MgCl2, 10 mM dithiothreitol, 1 mM ATP, and 20 U T4 RNA ligase (New England Biolabs, Beverly, MA) at 17°C overnight.
Ligated products were then washed in TE buffer, and amplified by exponential PCR using primer 2 that was partially nested relative to primer 1, and a ligation primer complementary to the ligation oligonucleotide. PCR was performed in a volume of 100 µl containing 20 mM Tris, pH 8.4, 50 mM KCl, 200 µM each dNTP, 1.5 mM MgCl₂, 100 nM each primer, and 2.5 U Taq DNA polymerase. The reaction mixture was heated at 94°C for 5 min, and subjected to 24 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min initially, with a 1 s extension for each subsequent cycle. An aliquot of 10 µl of a fresh mixture containing 45 nM primer 3 labeled with ³²P at its 5'-end, 20 mM Tris, pH 8.4, 50 mM KCl, 250 µM each dNTP, 1.5 mM MgCl₂, and 1 U Taq DNA polymerase was then added to the reaction tubes. An additional four cycles of PCR were performed, followed by termination at 72°C for 5 min and cooling to 4°C. The cycle numbers were within the exponential range of the PCR reaction for the sequences analyzed (data not shown). The magnetic beads were then removed from the reaction mixture, and the remaining products were precipitated with ethanol, electrophoretically separated in a denaturing 6% polyacrylamide gel, and analyzed by autoradiography or phosphorimaging.

Primer sequences were as follows. To assay for psoTFO1 adducts on the transcribed strand, primer 1 was 5'-biotin- d(CCTGTGCTCACAGATGAATTGG), primer 2 was d(TCATACAGATGAATTGGTTTCTCAAG), and primer 3 was d(GGT TTCTCAAGAATTCAGTAAAACC). To assay for psoTFO2 adducts on the non-transcribed strand, primer 1 was 5'-biotin-d(GAAGTGCCAAATGATACCTTATCCAT), primer 2 was d(CATAGATGACTATGCATGAT), and primer 3 was d(GAGACGTGAAATAGGGAGACAGAAGAG). The ligation oligonucleotide was dp(CTCGTAGATCATGATAGTCAGATCA- TA-C₃-amino) which represents an oligonucleotide that is 5'-phosphorylated and 3'-protected with a propylamine group to prevent formation of multimers. The ligation primer was d(TATGACTATGCTGATCTACGAT).

Epifluorescence microscopy

Cells grown on glass coverslips were transfected as described above with psoTFOs conjugated at their 3'-termini to FITC. After transfection, cells were washed once in DMEM and visualized with a Zeiss Standard Microscope 16 equipped with phase and epifluorescence condensers, a high pressure mercury excitation lamp, and filter sets appropriate for FITC.

Flow cytometry

Following transfection with FITC-conjugated psoTFOs, cells were trypsinized and washed once in PBS, then resuspended in PBS prior to analysis. Ten thousand cells were then analyzed with an EPICS Elite ESP flow cytometer (Coulter Inc., Fullerton, CA).

RESULTS

Design of oligodeoxyribonucleotides

Of the several polypurine sequences within the human collagenase gene that possess adjacent sites potentially reactive with psoralen, two were initially chosen by inspection to be most favorable. Figure 1 illustrates the relative orientation and position of each psoTFO within the human collagenase gene (17,18). Notably, both sites have a 5'-ApT-3' sequence adjacent to the 5'-end of the psoTFO. Although 5'-ApT-3' sites are typically minor sites for psoralens to bind and react, they have been successfully used as targets for psoTFOs (19). Oligonucleotides were designed using standard guidelines for Hoogsteen and reverse Hoogsteen base pairing that favor triple helix formation (20–23). psoTFO1 is a 19mer pyrimidine-rich sequence designed to bind parallel to the purine-rich transcribed strand at the start of exon 10; 5-methyl-deoxycytidine was used instead of deoxycytidine to enhance binding at physiological pH (24). Although the target strand for psoTFO1 is not entirely composed of purines, the exceptions (thymine and cytosine bases in the purine-rich strand) have been accommodated in the TFO with guanine and thymine, respectively, which have been successfully used before (20). psoTFO2 is a 17mer homopurine sequence designed to bind anti-parallel to the purine-rich non-transcribed strand within the intron between exons 6 and 7. psoTFO1 and psoTFO2 possessed in vitro dissociation constants (Kₘ) of 4 and 0.6 µM, respectively (25; manuscript in preparation).

Detection of targeted HMT adducts in cDNA sequences

A plasmid containing the human collagenase cDNA, pBScoll.dna, was linearized and treated with psoTFO1 or psoTFO2 for 2 h at room temperature, followed by 7 J/cm² UVA. Primer extension using Taq polymerase demonstrated the dose-dependent formation of a single truncated product corresponding to the site of psoralen adduct formation in the cDNA sequence treated with psoTFO1 (Fig. 3). The dependence of site-specific truncation of polymerization upon psoTFO1 concentration approximated the experimentally measured Kₘ of 4 µM (25; manuscript in preparation). Since psoTFO2 is designed to bind to an intron, it has no identifiable binding sites in the cDNA sequence, and did not form adducts at the psoTFO1 binding site, even at concentrations of up to 100 µM.

Detection of targeted HMT adducts in genomic DNA sequences

Since psoTFOs can in principle target photoadduct formation to a unique site in the genome, conventional methods of assaying psoralen adducts are not sufficiently sensitive to make detection practical at this lower limit of DNA damage. This is especially true when the target is a single copy gene, as is the interstitial collagenase gene (17,18). In addition, it is desirable to detect monoadducts in a strand-specific manner in order to understand the role of transcription in their processing; this is not a feature of any previously published assay for psoralen damage (12,26,27). Therefore, ssslig-PCR was adapted to detect psoTFO adducts in different strands of the native chromosomal collagenase gene in human cells. Purified genomic DNA from HT1080 fibrosarcoma cells was either untreated or treated with 5 µM psoTFO1, irradiated with 0.5 J/cm² UVA, and analyzed by ssslig-PCR. At this UVA exposure, the ratio of monoadducts to interstrand crosslinks is ~4:1 (manuscript in preparation). As shown in Figure 4A, untreated DNA produces only a single major band defined by the downstream restriction endonuclease site, as expected for undamaged DNA (lane 5). In contrast, samples treated with both psoTFO1 and UVA additionally exhibited a shorter product whose length corresponds to truncation of the polymerase reaction at the HMT...
adduct formed at the psoTFO1 binding site (lanes 1–4 and 6–8). No such truncation was seen in the absence of UVA, as expected since non-covalently bound psoTFOs would not remain bound to their targets during the denaturing conditions of this assay (data not shown).

To determine if the sslig-PCR assay is linear with respect to the amount of DNA and damage, psoTFO1-damaged genomic DNA was serially diluted prior to sslig-PCR. A linear relationship between the relative amount of initial DNA and the final amplified signal resulted (Fig. 4A, lanes 1–4, and B, top). Although this assay measured the absolute quantities of damaged and undamaged DNA reasonably well under ideal conditions, in routine practice the errors in estimating absolute DNA quantities as well as the multiple steps involved in the sslig-PCR assay produced considerable variation in absolute signal intensity. Therefore, the adduct fraction (the ratio of the adduct band intensity to the sum of adducted and undamaged DNA bands) was used as an alternative measure of DNA damage produced by the psoTFO. As shown in Figure 4B (top), this quantity was as high as 40% for psoTFO1 adducts under the experimental conditions employed and was invariant with the absolute DNA quantity. In similar experiments, 5 \text{M} \text{psoTFO2} irradiated with 0.5 \text{J/cm}^2 \text{UVA damaged its target with an adduct fraction of 15% (data not shown). As further confirmation that the assay would reliably measure a range of adduct fractions, serial dilution of the damaged DNA sample with undamaged DNA produced a linear relationship between the initial amount of damaged DNA and the sslig-PCR adduct fraction (Fig. 4A, lanes 5–8, and B, bottom).

Distribution of adducts produced by free HMT

Although psoTFO1 and psoTFO2 appeared to place photoadducts specifically at 5'-ApT-3' sites adjacent to their

intended target sequences, it was important to ascertain that a psoTFO site was not also associated with unusually high affinity for free HMT. One potential obstacle in using the sslig-PCR method for analyzing random crosslinking agents is that

Figure 1. Structure of psoTFOs and their targets in the human interstitial collagenase gene. HMT was linked to the oligonucleotide by either a two-carbon linker in psoTFO1 (n = 2) or a six-carbon linker in psoTFO2 (n = 6). Arrows with numbers indicate the position of the psoralen binding site with respect to the major transcription start site for the gene.

Figure 2. Schematic representation of single-strand ligation PCR to analyze photoadducts produced by psoTFOs. The example shown corresponds to a psoralen monoadduct targeted by the TFO to one strand of a double-stranded DNA target. Following digestion downstream of the target site by a restriction endonuclease, extension of a biotinylated primer proceeds to the restriction site in undamaged DNA, or prematurely terminates at the psoralen adduct. Extension products are captured with magnetic beads coated with streptavidin, and ligated at their 3'-termini to a defined oligonucleotide. Exponential PCR amplifies products that are separated and detected.
in order to form sufficient DNA damage within the short length of DNA assayed (<200 bp), the concentration of HMT adducts in the genome must be relatively large. Under these conditions, psoralen would form enough crosslinks to cause the genomic DNA to renature prior to annealing of PCR primers. If present in sufficient numbers, crosslinks might even prevent denaturation. Therefore, genomic DNA was treated with 100 µM HMT in TE for 1 h at room temperature, followed by irradiation with 390–450 nm light of varying doses; these wavelengths form monoadducts but do not convert them to significant numbers of interstrand crosslinks (28,29). As shown in Figure 5 (lanes 1–7), under these experimental conditions sslig-PCR was able to detect multiple adduct sites reflecting psoralen adducts that depended on the light dose. While it appears that the psoTFO1 target site is a minor site for reaction with free HMT, the primary target sites for free HMT adducts occur in a region ∼50 bp upstream of the intended psoTFO1 target. Comparison with the sequence of the collagenase gene reveals that this region upstream of the psoTFO1 target sequence is relatively rich in tandem TA sequences (17). In contrast, and as expected, sslig-PCR was unable to detect significant adduct formation in genomic DNA treated with 1, 10 or 100 µM HMT and irradiated with 0.1, 0.5, 1.0 or 5.0 J/cm² UVA doses (see for example Fig. 5, lane 8).

Efficient transfection of psoTFOs into human cells

psoTFO1 and psoTFO2 modified at their 3′-termini with FITC were used to monitor the distribution and kinetics of oligonucleotide entry into the cell. When incubated with cationic lipids for 2 h at 37°C, both HT1080 and XP12RO-SV40 exhibited bright nuclear fluorescence, and much weaker cytoplasmic fluorescence, as revealed by epifluorescence microscopy (Fig. 6). Flow cytometry showed that >90% of cells could be transfected in this manner, whereas no significant transfection occurred in the absence of the lipid carrier.

Detection of targeted psoralen adducts in human cell lines

To measure the efficiency of psoTFO targeting in human cells, initial experiments were performed in a repair-deficient cell line.
line from the XPA complementation group to ensure that no adducts would be lost as a consequence of excision repair. As seen in Figure 7, XP12RO-SV40 cells treated with 250 nM psoTFOs and UVA displayed bands corresponding to the expected site-specific adducts at both the psoTFO1 (lanes 2 and 3) and psoTFO2 regions (lanes 11 and 12). The adduct fraction in psoTFO1-treated cells under these conditions was 15%. Due to extensive background, the adduct fraction for psoTFO2 was difficult to quantitate, but since it was necessary to use exposures that saturated the bands corresponding to undamaged DNA in order to clearly see the psoTFO2 adduct, the adduct fraction is probably smaller for psoTFO2 than for psoTFO1. Using sslig-PCR, we were unable to detect binding of psoTFO1 to the psoTFO2 region (lanes 5 and 6) or of psoTFO2 to the psoTFO1 region (lanes 8 and 9). Although a number of other minor bands appeared in the sslig-PCR reaction, these were present even in the absence of the psoTFOs and they therefore represent the non-specific background of the assay. Addition of TPA prior to transfection to increase transcription through the collagenase gene did not noticeably alter the pattern of adduct formation or the adduct fraction in either of these regions (lanes 3, 6, 9 and 12).

When repair-proficient HT1080 cells were similarly treated with psoTFO1, similar levels of adducts (~15%) were also observed immediately after UVA irradiation (Fig. 8, lane 3). No adducts were seen in cells that were irradiated in the absence of psoTFO1 (lane 2).

DISCUSSION

The ability of TFOs to bind to specific DNA sequences has generated considerable interest because it offers a means of selectively altering gene expression, either as a steric block to DNA metabolism or as a mutagen (1–3,30,31). Most experimental studies using TFOs have targeted DNA sequences in plasmids or other exogenous vectors. However, in order to fulfill their potential, TFOs must be able to target sites in native chromosomal DNA within a mammalian cell, because chromatin structure and dynamics play significant roles in determining target accessibility and in processing the resulting lesion (32,33). Site-specific mutations and deletions in chromosomal DNA have been attributed to triple helix formation in mammalian cells (4,5), but only a few studies have attempted to provide evidence that TFOs actually bind to native chromosomal DNA target sequences within living mammalian cells. In one case, protection of DNase I hypersensitive sites within the c-myc gene in association with TFO treatment suggested binding to the target sequence (34); however, an alternative explanation for this result has been proposed whereby TFOs compete for transcription-activating factors that are associated with DNase I hypersensitivity (35). In other cases, it has been necessary to use agents such as streptolysin O and digitonin that permeabilize the cellular membrane with physical pores in order to transfact TFOs successfully into cells so that their binding could be directly observed (6,7). Moreover, interpretation of previous binding studies is potentially complicated by the observation that TFOs not bound to their targets in cells can persist and co-purify with the genomic DNA, and subsequently bind to their targets, producing results mimicking intracellular binding, unless explicit steps are taken to remove the initially unbound TFOs (8). Our results presented here directly demonstrate that psoTFOs bind to their targets in cell-free and intact intracellular environments.
As an extension of its ability to bind short synthetic DNA duplex targets (manuscript in preparation), psoTFO1 binds in vitro to longer collagenase cDNA sequences, placing a specific psoralen photoadduct at an intended site that is detectable by a primer extension assay. This interaction is specific since psoTFO2 does not block primer extension in the same region, even though multiple 5′-TpA-3′ and 5′-ApT-3′ sequences exist which are potential psoralen binding sites in the region assayed and psoTFO2 is weighted toward binding purine-rich sequences. Because the results presented are for one target strand only, it is not yet possible to determine if the photoadducts detected by this assay exclusively represent monoadducts, or include crosslinks as well. Interstrand crosslinks will prevent the individual strands of the duplex DNA target from completely dissociating, and it has been previously observed that renaturation of the target can effectively compete with primer annealing (36).

In order to detect and reliably measure the level of damage created by psoTFOs in cells, it was desirable to use an assay that would be sensitive and avoid binding artifacts. Ligation-mediated PCR has been used to detect TFO delivery of an alkylating agent, although this approach exploited the depurination and cleavage reactions specially associated with alkylating damage (7,37). In contrast, sslig-PCR is in principle a completely general method for detecting any DNA lesion capable of blocking a DNA polymerase, and although it has been used to detect cis-platinum adducts at the nucleotide level, it has not yet been used to assay psoralen or psoTFO adducts (15,16). Moreover, because this assay generates its initial primer extension products at temperatures that would denature non-covalently bound psoTFOs and then removes genomic DNA and any unreacted oligonucleotides at a very early stage, the probability of spurious binding from residual, unreacted psoTFOs is diminished. For the psoTFOs targeted to the collagenase gene, sslig-PCR responds linearly to the amount of damaged DNA, and the adduct fraction reliably reflects the relative amount of DNA damage. Under the conditions of 5 µM psoTFO1 and 0.5 J/cm² UVA, the site-specific adducts represented 40% of the signal. As discussed above, because the first step of the sslig-PCR assay is primer extension, interstrand crosslinks will be under-represented or undetectable, while monoadducts should be reliably detected.

It was important to determine if HMT unconjugated to its TFO would be capable of generating adducts similar to that produced by the psoTFO. Such a situation might arise, for example, if HMT was intracellularly hydrolyzed from its TFO, or if the binding ability of HMT dominated over that of the TFO. As discussed above, the adducts created by free HMT irradiated with the UVA doses used in these experiments are extremely poorly detected by sslig-PCR, probably due to rapid
renaturation of the duplex target which competes with primer annealing. Visualization of HMT binding sites necessitates creation of predominantly monoadducts using long wavelength irradiation. Under these conditions, multiple HMT adducts occurred in the region assayed by ssig-PCR; although the psotFO1 binding site was weakly represented, the most prominent signal was due to a TA-rich sequence ~40–50 bp upstream of the psotFO1 target. This distribution concurs with previous work which has shown a preference for psoralen adduct formation in TA-rich sequences (38). These experiments indicate that even if the HMT of the psotFO did interact independently of the TFO, the resulting adducts would have a markedly different distribution from the TFO-directed adduct, and they would be extremely difficult to detect under normal experimental conditions. Therefore, psotFOs are capable of normally reactive, and it is extremely unlikely that the signals seen by ssig-PCR are due to random HMT damage.

Although transfection of cells with oligonucleotides is routinely accomplished to suppress gene expression by antisense mechanisms, chromosomal DNA adduct formation in cells by TFOs is still a formidable task because sufficient quantities of TFOs must enter the nucleus intact and be free to interact with a DNA target that may be masked by other chromosomal components. In the present work, we were able to transf ect repair-deficient XP12RO-SV40 human fibroblasts efficiently with psotFOs using cationic liposomal preparations that allowed accumulation of psotFOs in the cellular nuclei. Upon irradiation, genomic DNA from treated cells showed adducts at the appropriate target sequences for the psotFO, indicating that TFOs had reached the appropriate nuclear compartment, had bound to the target sequence, and allowed the HMT moiety to photoreact. Because ssig-PCR likely underestimates the number of crosslinks, the measured adduct fractions represent lower limits of damage in these cells with the UVA and psotFO dosages used. Interestingly, psotFO1 appears to form a higher adduct fraction in cells than psotFO2, in spite of a poorer affinity in vitro (manuscript in preparation). The measured adduct fraction of 15% produced by 250 nM psotFO1 transfected by cationic lipid is somewhat higher than the ~10% reported for 10 µM psotFOs with phosphodiester backbones targeted to the HIV polypurine tract of HIV-1 integrated into chromosomes of U1 cells (6). These results collectively suggest that neither the accessibility of psotFO targets nor the persistence of the non-covalently bound psotFO is influenced by transcription. Because SV40 transformation has been shown to up-regulate basal collagenase gene transcription specifically relative to that of other matrix metalloproteinases in human skin fibroblasts, one might expect that additional stimulation by TPA would not significantly affect accessibility (39). On the other hand, TPA still appears to induce collagenase mRNA levels above SV40-transformed levels (39). Although prolonged mRNA lifetimes could contribute to increased mRNA levels in TPA-stimulated SV40-transformed fibroblasts (40), the effects of TPA on transcriptional activation of the collagenase gene are well known (41), and any up-regulation of transcription would imply that non-covalently bound psotFOs are able to persist at their targets in spite of the increased transcriptional activity prior to UVA irradiation.

The lifetime of an individual repair event mediated by nucleotide excision repair has been estimated to be of the order of minutes (42). In principle, therefore, a single psotFO adduct targeted to a unique site in a cellular genome, if recognized immediately, could potentially be efficiently removed by a repair-competent cell during the period following UVA irradiation and before cell lysis. Therefore, repair-proficient HT1080 cells were examined for psotFO adducts following UVA irradiation. These cells display psotFO1 adducts at similar levels to those of XP12RO-SV40, suggesting that dramatic repair of these particular adducts does not occur within minutes, the time required to lyse the cells following UVA irradiation.

The psotFOs employed in the current work bind several orders of magnitude less strongly to their duplex targets than observed for some other previously reported TFOs which have demonstrated nanomolar dissociation constants in vitro and site-specific mutagenesis in mammalian cells (3,43). Because of the relatively weaker affinities for their targets, it is intriguing that psotFO1 and psotFO2 adducts are formed to any significant degree within cells. However, psotFO1 is a pyrimidine-rich oligonucleotide, and psotFO2 is a purine-rich oligonucleotide that is relatively guanine-poor. At the expense of poorer molecular affinity, the absence or paucity of guanine in these sequences may make the intracellular formation of unusual tertiary or quaternary structures less likely, allowing larger quantities of psotFO to be accessible for binding, in contrast to other TFOs that are relatively rich in guanine residues and that have exhibited binding or mutagenesis in cells (6,7,43,44). Additionally, cationic lipid-mediated concentration of psotFOs within the nucleus of the cell may in part offset problems of weaker in vitro binding. Unlike methods in which equilibrium extracellular concentrations of TFOs are introduced into cells by agents that result in physical permeabilization of the cell (4,6,7), cationic lipid vectors may direct and concentrate psotFOs more efficiently to the proper cellular compartment for binding to the chromosomal target. A final possibility to account for differences among various experiments with TFOs is that different DNA targets may not be equally accessible to TFO binding, independent of the in vitro measurements, as seen in comparing psotFO1 and psotFO2...
REFERENCES

1. A. K. Ganas and C. A. Smith for helpful discussions and critical reading of the manuscript, W. Hoeffer for providing the human collagenase cDNA, J. Hartley and P. McHugh as well as S. Tornaletti for helpful advice and critical reading of the manuscript, W. Hoeffler for its activity in normal and pathophysiological states. When overexpressed, the gene participates in the pathophysiology of a variety of diseases, including arthritis, local invasion and distant metastasis of solid tumors, vascular aneurisms, chronic wounds and blistering diseases, and photoaging. Although it is an appealing pharmacological target and various exogenous agents are known that suppress its activity, there are few targets for TFOs. Collagenase is the only member of the matrix metalloproteinase family capable of performing the initial proteolytic cleavage in type I collagen, and thus it plays a central role in connective tissue homeostasis. Our results with two distinct regions of the collagenase gene in transformed human fibroblast-derived cell lines support the concept of using exogenous agents to specifically target the transcriptional activity of the interstitial collagenase gene (45,46). Our results with TFOs suggest that they have the potential to be useful pharmacological agents in specifically damaging the native cellular interstitial collagenase gene and in modifying its activity in normal and pathophysiological states.

ACKNOWLEDGEMENTS

We thank A. K. Ganas and C. A. Smith for helpful discussions and critical reading of the manuscript, W. Hoeffer for providing the human collagenase cDNA, J. Hartley and P. McHugh as well as S. Tornaletti for helpful advice and protocols on ssS-PCR, S. Sherwood for assistance with cell sorting, and J. Berry for use of a visible light photometer. This work was supported by Mentored Clinical Scientist Development Award K08-AR02008 from the National Institute for Arthritis, Musculoskeletal and Skin Diseases (D.H.O.) and Outstanding Investigator Grant CA44349 from the National Cancer Institute (P.C.H.).

REFERENCES


Downloaded from https://academic.oup.com/nar/article/27/24/4734/2902064 by guest on 22 March 2024