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Triplex targeting of a native gene in permeabilized intact cells: covalent modification of the gene for the chemokine receptor CCR5

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ABSTRACT

A 12 nucleotide oligodeoxyribopurine tract in the gene for the chemokine receptor CCR5 has been targeted and covalently modified in intact cells by a 12mer triplex forming oligonucleotide (TFO) bearing a reactive group. A nitrogen mustard placed on the 5′-end of the purine motif TFO modified a guanine on the DNA target with high efficiency and selectivity. A new use of a guanine analog in these TFOs significantly enhanced triplex formation and efficiency of modification, as did the use of the triplex-stabilizing intercalator coralyne. This site-directed modification of a native chromosomal gene in intact human cells under conditions where many limitations of triplex formation have been partially addressed underscores the potential of this approach for gene control via site-directed mutagenesis.

INTRODUCTION

Site-directed mutagenesis of genomic DNA offers a viable alternative to vector-based gene therapy in instances where the gene modification can be precisely targeted. This could be accomplished by targeting a specific sequence on DNA with an oligonucleotide bearing an electrophilic group. Reaction of that group with a targeted nucleotide on DNA would induce mutations at that site. The use of triplex-forming oligonucleotides (TFOs) as the addressing component could provide an appealing route to this goal (1,2). Photoreactive TFOs have been used to cause site-directed mutations in a shuttle vector system in cells (3–5). More versatility in targeted modification can be provided by TFOs bearing electrophilic agents which, by design, alkylate targeted nucleotides (2,6–10). The use of TFOs to achieve specific covalent modification of DNA may be a preferred application, since reversible complexes of TFOs which target double stranded DNA may be too weak to affect gene function directly. Only very recently, in fact, has triplex formation in native chromatin structure been directly demonstrated (11).

An attractive application of this approach to gene modification is the chemokine receptor CCR5 (12), known to serve as a co-receptor for uptake of macrophage-tropic strains of HIV into $CD4^+$ cells (13). An inherited mutation in this gene, Δ32, has been shown to abrogate functional expression of the gene, and individuals homozygous for the mutation are apparently immune to HIV infection (14,15). The functional expression of the gene does not appear to be critical in healthy humans, and induction of a mutation via a site-directed modification may have the same protective outcome. There is a short homopurine tract in the CCR5 gene that is 12 bases long, normally insufficient for effective TFO binding. This length issue has been overcome by modifications which significantly enhance binding of the TFO. The new TFOs used the G/A motif (16–18), with all guanines replaced by an isosteric guanine analog. We show here, using ligation-mediated PCR (LMPCR), the direct covalent modification of targeted nucleotides in the CCR5 gene via triplex formation.

MATERIALS AND METHODS

Synthesis of oligonucleotides and conjugates

All oligodinucleotides (ODNs) were prepared from 1 μmol of the appropriate CPG support on an ABI 394 (Perkin-Elmer) using the protocol supplied by the manufacturer. Protected β-cyanoethyl phosphoramidites of 2′-deoxynucleosides, CPG supports, deblocking solutions, cap reagents, oxidizing solutions and tetrazole solutions were purchased from Glen Research. The guanines in TFO2 were replaced by 6-aminopyrazolo[3,4-*d*]pyrimidine-4(3*H*)-one (8-aza-7-deazaguanine, or ppG), using a phosphoramidite prepared as described by Seela and Driller (19). The aminohexyl modification at the 5′-end of the precursor of the TFOs was introduced using an *N*-(4-monomethoxytrityl)-6-amino-1-hexanol phosphoramidite linker (Glen Research). A 3′-hydroxyhexyl phosphate was incorporated into each TFO using a modified CPG (20). All other general methods employed for ammonia de-protection, HPLC purification, detritylation and butanol precipitation of ODNs were carried out using standard procedures as previously described (20). ODNs were >95% pure by C-18 HPLC and formed one major band by capillary gel electrophoresis, which was performed on a P/ACE 2000 Series equipped with an eCAPTM cartridge (Beckman, Fullerton, CA).

The 5′-aminotailed TFOs were conjugated with the 2,3,5,6-tetra-fluorophenyl esters of either chlorambucil or phenylacetate mustard as described (21). They were isolated from reaction mixtures by HPLC with 50–70% yield. To maintain >90% alkylating activity of the TFO, all manipulations with collected HPLC fractions, including concentration with butanol, precipitation and washing with acetone, were performed in ice-cold solutions. The reactive TFO was dissolved in water and stored at –70°C.

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The integrity of the conjugated nitrogen mustard was assessed by reported assays (21). The purified conjugates were analyzed by C-18 HPLC (column 250 × 4.6 mm) in gradient of 5–45% acetonitrile in 0.1 M triethylamine (TEA) acetate buffer (pH 7.0) over 20 min at a flow rate of 1 ml/min. Pump control and data processing were performed using a Rainin Dynamax chromatographic software package on a Macintosh computer. The reactive TFO conjugates were >90% pure by C-18 HPLC.

Model crosslinking reactions

Crosslinking, cleavage and sequencing reactions were performed on a 60mer synthetic duplex target as described (10,22). The reaction mixture contained 20 nM labeled duplex, 2 μM TFO and 8 μM coralyne in 140 mM KCl, 10 mM $MgCl_2$, 1 mM spermine and 20 mM HEPES (pH 7.2), incubated at 37°C for 4 h.

Isolation of genomic DNA and reaction with TFO

Genomic DNA from HT-29 adenocarcinoma cells (ATCC# HTB-38) was prepared with a Wizard Genomic DNA Purification Kit (Promega) using the protocol supplied by the manufacturer. To a solution of 5–10 μg of genomic DNA in 90 μl 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 140 mM KCl, 10 mM $MgCl_2$, 10 mM HEPES (pH 7.2) and 1 mM spermine was added 10 μl of a 10× stock of the TFO to give a final ODN concentration of 2 μM. After mixing and incubation overnight at 37°C, the DNA was precipitated with EtOH and dried, and LMPCR was conducted as described below.

Cell transfections

HT-29 cells were plated into a six-well 35 mm plate at 4.0×10^5 cells per well and were allowed to adhere for 4 h at 37°C in complete media. Then they were washed with PBS and treated for 5 min at 37°C with 350 μl of permeabilization buffer [137 mM NaCl, 100 mM PIPES (pH 7.4), 5.6 mM glucose, 2.7 mM KCl, 2.7 mM EGTA, 1 mM ATP, 0.1% BSA] containing 500 U/ml streptolysin O (pre-activated for 15 min at room temperature in the presence of 2.5 μM DTT), 8 μM coralyne and TFO2. Five ml complete media were then added and cells were incubated for another 6 h at 37°C. Cells were then thoroughly washed with PBS, to remove dead cells and cell debris, and then trypsinized. The DNA was isolated as above and subjected to LMPCR as below.

Ligation-mediated PCR

Most steps of this technique were performed as described by Pfeifer and Riggs (23) and Meuller *et al*. (24) with two modifications. The first modification was generation of an internal control site by restriction of the DNA after treatment with the reactive TFO (25). An *Eco*RI site 5′ to the targeted base on the non-coding (pyrimidine) strand was used, as shown in Figure 1. The DNA samples were then restricted to completion by incubating 3 h under optimal conditions with a 3-fold excess of restriction enzyme. The volume was adjusted to 100 μl with water and, after restriction, the DNA was ethanol precipitated. The pellet was resuspended in 10 mM Tris–HCl (pH 7.5) and 1 mM EDTA, to give a DNA concentration of ~0.5 μg/μl. To 5 μl of this chilled solution in a PCR tube was added 25 μl of the first strand synthesis solution (24). The second modification was heating the treated DNA at 95°C in this first strand synthesis buffer, pH 8.9, for 10 min prior to annealing and extending the first primer. This caused quantitative depurination and cleavage of the DNA at any site of base alkylation. First strand synthesis and ligation of the universal linker was performed as described (24), followed by nested PCR (23). Phosphorimaging was used to analyze the electropherogram of the LMPCR results, and the intensity ratio (alkylation)/(alkylation + restriction) gave the efficiency of targeted alkylation. We have recently shown that this method can reliably quantify the efficiency of nucleotide-specific DNA targeting (25).

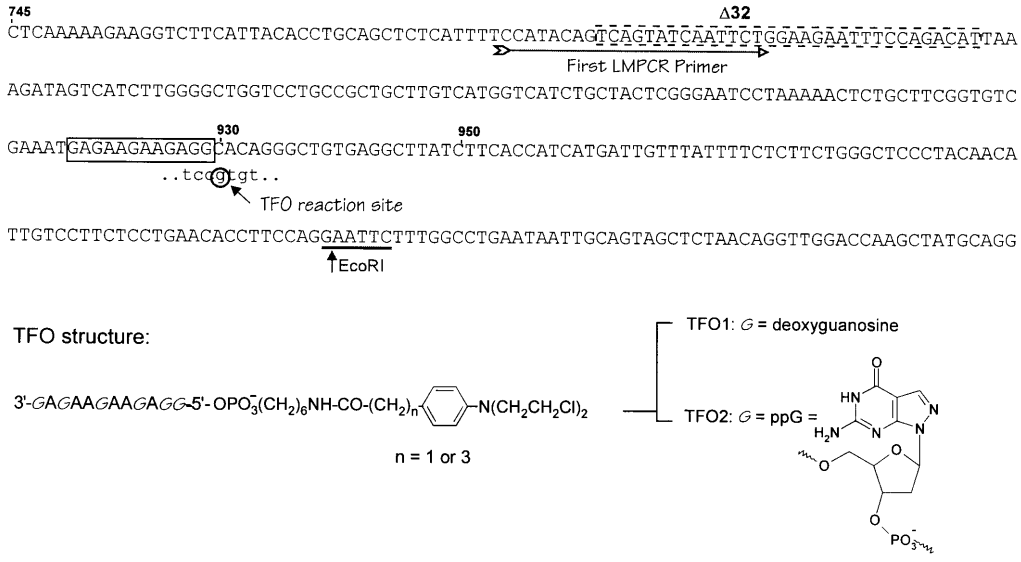

Figure 1. Sequence of the targeted region of the CCR5 gene and structure of the TFOs used in this study. The portion of the sequence shown (12) begins at nucleotide 745, showing the region amplified by LMPCR, the sequences of primers, site of covalent modification (on the complementary strand), and restriction site. The TFO binding site and the Δ32 mutation are indicated.

RESULTS AND DISCUSSION

The targeted portion of the CCR5 gene and the position of the Δ32 mutation are shown in Figure 1. The reversible binding of the TFO 12mer core sequence to the 12 nucleotide homopurine target site was weak; no triplex formation was seen in a non-denaturing gel mobility shift assay at pH 7.2, 37°C (data not shown). Measurement of triplex-directed target alkylation (affinity labeling) was therefore used to quantitate the ability of the TFO to form a sequence-specific complex. Affinity labeling has previously been used to measure triplex formation by TFOs with appended electrophilic (26) and photocrosslinking groups (27). Two 12mer G/A TFOs, antiparallel to the purine tract of the coding strand, bearing the nitrogen mustard chlorambucil on the 5′-terminus were prepared. These TFOs differed in the nature of the guanine bases present: TFO1 contained normal guanines, but in TFO2 all guanines were replaced with ppG (19), which we have found to give a significant increase in the rate of triplex formation and some increase in triplex stability (unpublished results). These TFOs were designed to alkylate the *N*7 of the guanine on the non-coding strand opposite C-930. In these experiments, we used coralyne, a cationic intercalator previously shown to stabilize triplexes of the C/T (28) and G/A (22,25) motifs, which we have found also stabilizes triplexes in cells. We have also previously found that concentrations of coralyne <10 μM are well tolerated by a number of tested cell lines and do not give any DNA cleavage under any of the conditions we use in our assays (data not shown).

Figure 2 shows the efficiency and specificity of these reactive TFOs in targeting a 60 bp synthetic model duplex. The DNA target strand containing homopyrimidine run (the non-coding strand of the CCR5 gene) was predominantly alkylated. We have found that a guanine in the pyrimidine strand, adjacent to the triplex site, is the preferred site of reaction for either of the nitrogen mustards used in this study when conjugated to the terminus of the TFO (10,22). Alkylation of the purine-rich strand did not exceed 2–6%. TFO1, consisting of normal purines, revealed 25% cross-linking in presence of 8 μM coralyne (Fig. 2A). Lower coralyne concentrations of 5 and 1 μM decreased the yield of target alkylation to 14 and 1%, respectively. No traces of the reaction were seen in absence of this triplex stabilizing intercalator. Substitution of all guanines by ppG gave a significant effect on reaction efficiency (Fig. 2B). TFO2 (Fig. 1, $n = 3$) showed 2% target alkylation in the absence of coralyne, and a high level of target alkylation of 74% with 8 μM coralyne. Increases in triplex stability by coralyne have been noted (22,28). The significant enhancement in triplex formation in a physiological buffer in these purine motif TFOs by the substitution of the ppG analog for guanine is new, however, and proved to be important in our ability to target CCR5. The positive effects on triplex formation by this analog contrast with the detrimental effects observed earlier for the substitution of 7-deazaguanine for the guanine base (29). The origin and scope of this effect is under investigation.

The identity of the targeted guanine in the pyrimidine strand was clarified by mapping experiments for both TFOs used in this study (Fig. 2C). The guanines predicted to react by our previous work (10,25) were the only sites of reaction, showing that these TFOs are indeed affinity labels and that the electrophiles do not react at any of the other guanines in the target.

Although mustards used in this study are bifunctional alkylating agents, we rarely see more than a single alkylation event on the target strands with these conjugates. In physiological conditions

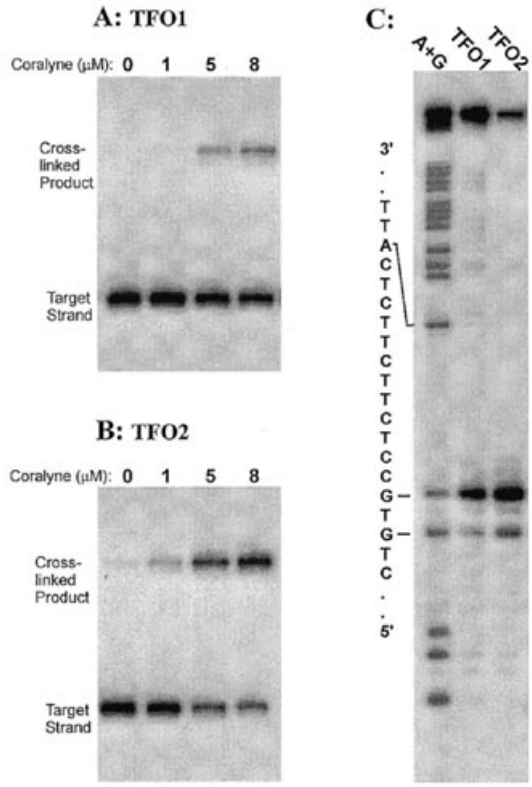

Figure 2. Efficiency and specificity of triplex-directed alkylation of a 60 bp model duplex (nucleotides 891–950 of the CCR5 gene, Fig. 1) by TFO1 ($n = 3$) and TFO2 ($n = 3$). The pyrimidine (non-coding) strand was ^{32}P-labeled. Incubations were performed for 4 h at 37°C in 140 mM KCl, 10 mM $MgCl_2$, 1 mM spermine and 20 mM HEPES (pH 7.2) as described in the Materials and Methods. The gel buffer was 90 mM Tris–borate, 20 mM EDTA and 8 M urea (pH 8.3). (**A** and **B**) Products from TFO1 and TFO2, respectively, as a function of coralyne concentration. (**C**) The site of alkylated bases as shown by cleavage fragments resulting from heat/piperidine treatment of the reactions using 8 μM coralyne, compared with an A+G sequencing ladder.

the *N*-(2-chloroethyl) groups react in the time frame of 1–3 h (21), and longer incubation had almost no effect on the yield of the cross-linking reaction. Since the mustards decompose at a fixed rate, we assume that, in these experiments, the degree of triplex formation, or site occupancy, is reflected in the degree of alkylation.

The reaction of TFO2 (Fig. 1, $n = 3$) with isolated human genomic DNA, as assessed with LMPCR, is shown in Figure 3. The first primer, used to prime the non-coding strand, is indicated in Figure 1. In work not shown here, we found that one CCR5 allele of these HT-29 cells has the Δ32 mutation. The single *Eco*RI band from the primer results from the fact that the primer site overlapped the Δ32 site, hence displaying only those reactions on the wild-type CCR5 allele. TFO2 gave almost quantitative modification of the targeted genomic DNA site at 2 μM in the presence of 8 μM coralyne. Without this triplex stabilizer, only trace target alkylation was seen by LMPCR. As expected from the data in Figure 2, and as predicted by our previous study on genomic DNA targeting by reactive TFOs (25), the only site of significant alkylation was the predicted target site, indicating triplex-directed reaction.

Triplex-mediated targeting of the native CCR5 gene in intact cells is shown in Figure 4. The oligonucleotide used for this

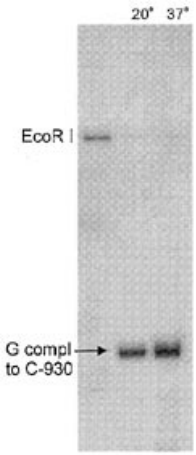

Figure 3. Modification of the CCR5 gene in isolated genomic DNA. The first LMPCR primer site is shown in Figure 1. The left lane is the control reaction with no TFO added, and the two other lanes are labeled with the temperature at which the hybridization reaction was conducted. Gel conditions are as in Figure 2. The pyrimidine strand was amplified after TFO2 (n = 3, 2 μM) treatment for 4 h.

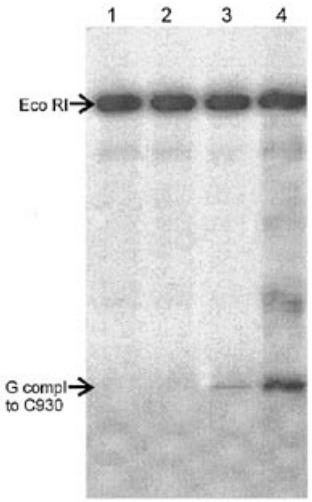

Figure 4. Modification of the CCR5 gene in cells. After treatment of cells with phenylacetate mustard-conjugated TFO2 (Fig. 1, n = 1), DNA was isolated and the pyrimidine strand was amplified as described in Material and Methods. Gel conditions are as in Figure 2. Lane 1, no TFO added; lane 2, non-targeted sequence TFO with conjugated phenylacetate mustard, 5 μM; lane 3, 5 μM TFO2; lane 4, 20 μM TFO2. The non-targeted sequence used in lane 2 is 5′-AGGAGAAAGGA-GAGGAGAGAG with the 5′-phenylacetate mustards conjugated as in TFO2.

experiment was TFO2 with a phenylacetate mustard conjugated (Fig. 1, n = 1). This mustard has a slightly longer half-life (21) and gives somewhat better modification efficiency in cells than chlorambucil. The TFO was also modified with a 3′-hydroxyhexyl phosphate to slow the rate of endonuclease digestion (20). HT-29 cells were treated with this TFO in the presence of 8 μM coralyne and streptolysin O, used to render the cells permeable to an ODN (30,31). This treatment has been shown to leave cells permeable to macromolecules while retaining their viability under appropriate conditions (32). To ensure that the observed modification was not due to modification of free DNA from dead cells, all cell debris was carefully removed prior to workup of the cells. Furthermore, the phenylacetate mustard used here has a half-life of ~50 min at 37°C (21), and the incubation time (6 h) was greater that six half-lives. After this time, the mustard would be almost completely decomposed. The site-directed labeling efficiency, as quantified by comparison with the *Eco*RI restriction site, was 5% at 5 μM and 24% at 20 μM TFO2. Bands occurring between the *Eco*RI restriction fragment and the cleavage site are technique artifacts and occur in every lane. Both coralyne addition and the use of the modified guanine analog ppG in this TFO were important for obtaining the level of genomic modification seen.

These results demonstrate site-directed covalent modification of a native mammalian gene in intact cells by a phosphodiester ODN with an electrophilic reactive group attached. This is direct evidence of effective formation of a triplex in an endogenous gene within the native chromatin structures found in cells. Covalent modification of specific sites in genes, especially as more versatile targeting methods are developed, will ultimately allow the introduction of designed mutations to alter gene function in an inheritable fashion. An agent that could eliminate functional expression of CCR5 in lymphocytes, much as Δ32 does, would provide a unique approach for the study of this co-receptor in initiation and progression of HIV infection.

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