Repairing the Sickle Cell Mutation

I. SPECIFIC COVALENT BINDING OF A PHOTOREACTIVE THIRD STRAND TO THE MUTATED BASE PAIR*

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A DNA third strand with a 3′-psoralen substituent was designed to form a triplex with the sequence downstream of the T-A mutant base pair of the human sickle cell β-globin gene. Triplex-mediated psoralen modification of the mutant T residue was sought as an approach to gene repair. The 24-nucleotide purine-rich target sequence switches from one strand to the other and has four pyrimidine interruptions. Therefore, a third strand sequence favorable to two triplex motifs was used, one parallel and the other antiparallel to it. To cope with the pyrimidine interruptions, which weaken third strand binding, 5-methylcytosine and 5-propynyluracil were used in the third strand. Further, a six residue “hook” complementary to an overhang of a linear duplex target was added to the 5′-end of the third strand via a T4 linker. In binding to the overhang by Watson-Crick pairing, the hook facilitates triplex formation. This third strand also binds specifically to the target within a supercoiled plasmid. The psoralen moiety at the 3′-end of the third strand forms photoadducts to the targeted T residue and, when this target is contained in a linear duplex fragment, a duplex-forming hook (Fig. 1b) to provide sufficient third strand binding energy to enable triplex-mediated psoralen photoaddition in high yield precisely at the mutant base pair.

EXPERIMENTAL PROCEDURES

Deoxynucleotides—These were synthesized by automated phosphoramidite chemistry, purified to homogeneity by denaturing PAGE, and recovered from gel slices via the “modified crush-and-soak” method (11). Final purification was by acetonitrile/water (50/50) elution from C18 Sep-Pak reverse phase columns (Millipore), followed by spin evaporation to dryness. Oligomer concentrations were adjusted spectrophotometrically in Milli-Q-purified water. Psoralen was attached to the 3′-end of designated oligomers by incorporating the phosphoramidite of psoralen CPG (containing a C-16 linker between psoralen C-3 and DMT) (Chemgenes Corp., Waltham, Massachusetts) during oligomer synthesis. Oligomer homogeneity was ascertained by32P-5′-end-labeling and denaturing PAGE. Oligomers were 3′-P-5′-end-labeled preparatively by incubating approximately 4 pmol of oligomer with 2 mCi [γ-32P]ATP (Amersham Pharmacia Biotech), and 1 unit of T-4 polynucleotide kinase (U. S. Biochemical Corp.) at 37 °C for 1 h, purified by denaturing PAGE, and eluted from C18 Sep-Pak columns.

Buffers—All experiments, unless otherwise noted, were performed in 0.1 M NaOAc, pH 5.0, 0.01 M Mg(OAc)2 (standard buffer).

Triplexes—Duplexes were formed by mixing equimolar amounts of each strand, heating to 80 °C and slowly annealing to room temperature in the standard buffer. Various ratios of third strand were added to pre-formed duplex, and heating mixtures were incubated for 1 h at room temperature, and then at 4 °C overnight.

Electrophoresis—Denaturing PAGE was performed on slabs 25×45 cm using 8 M urea, TBE, 16% polyacrylamide gels (1:37 bisacrylamide/acrylamide). Samples were dissolved in denaturing loading buffer (12) and heated to 80 °C before loading. Denaturing gels were run for 2–6 h at 1.5 kV at room temperature. Nondenaturing PAGE was conducted on a 20×20-cm slab of 15% polyacrylamide in the standard buffer (see above) and run at 4 °C for 12 h at 150 V. Denaturing gels were soaked in standard fixing solution (12), then in 100% methanol to homopyrimidine target sequences 15–25 base-pairs long occur with limited frequency. The second barrier is that in the face of imperfect targets, third strand binding energy is often insufficient to permit stable triplex formation.

We have exploited the triplex-based approach to develop a strategy for correcting the mutation that underlies human sickle cell anemia, which is due to an AT → TA transversion in the gene for the β-globin chain located on chromosome 11 (9). This mutation occurs immediately upstream of a 24-nt purine-rich sequence that consists of two adjacent regions located on opposite strands, the longer downstream one containing four base pair inversions. These encumbrances make it a difficult target for third strand binding, requiring a strand-switching “cross-over” strategy (10), the use of modified third strand residues and, when this target is contained in a linear duplex fragment, a duplex-forming hook to provide sufficient third strand binding energy to enable triplex-mediated psoralen photoaddition in high yield precisely at the mutant base pair.

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* This work was supported in part by fellowships from Codon Pharmaceutical, Inc. and Oncor, Inc. (to O. A. and N. G. D., respectively), and by Grant DE-FG02-96-ER62202.A001 from the Department of Energy. A mutant base pair of the human sickle cell β-globin chain (11, 12) is targeted with a 24-nt purine-rich sequence that consists of two adjacent regions located on opposite strands, the longer downstream one containing four base pair inversions. These encumbrances make it a difficult target for third strand binding, requiring a strand-switching “cross-over” strategy (10), the use of modified third strand residues and, when this target is contained in a linear duplex fragment, a duplex-forming hook to provide sufficient third strand binding energy to enable triplex-mediated psoralen photoaddition in high yield precisely at the mutant base pair.

1 The abbreviations used are: nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis; D-1/D2, coding strand of target duplex; D-2/D4, noncoding strand of target duplex; PsT-1, third strand without hook; pr 7U, 5-propynyluracil; me5C, 5-methylcytosine; PsT-2, third strand with hook; PsT-3, third strand with hook and scrambled triplex-forming sequence.


Binding of a Third Strand to the Sickle Cell Gene Target

**Fig. 1. Oligonucleotide structures.**

- **a.** Triplex structure formed by association of third strand PsT-1 and the duplex segment of β-globin gene D_{1}-D_{2} at the third strand binding target. The A→T transversion mutation site on D_{1} is indicated in bold letters and by an arrow. PsT-1 contains modified residues (°C = me°C, °U = pr°U) in the triplex-forming region that binds to D_{2} in the pyrimidine/parallel motif and then crosses over to bind to D_{1} in the GT/antiparallel motif, terminating with a 5'-psoralen moiety positioned opposite the mutant residue T_{11} on D_{1}. An acridine intercalator moiety is added to the 5'-end of PsT-1 to strengthen third strand binding. b, triplex structure formed by association of strand PsT-2 and duplex D-1-D-2, which are shortened versions of D_{1} and D_{2}, respectively. The 3'-end of D_{1} is truncated by 6 nt to create a target on D-2 for a duplex-forming hook linked to PsT-1 to create third strand PsT-2. The hook, complementary to the 5'-end of D-2, is followed by a (T)_{4} linker and then the same triplex-forming sequence containing modified residues (°C = me°C, °U = pr°U), that binds to duplex segment C. PsT-2 then crosses over to bind along strand D-1 of duplex segment B, terminating with a 3'-psoralen moiety positioned opposite the mutant residue T_{11} on D-1. Duplex segment A should not be protected from nuclease digestion by third strand binding, whereas segments B and C are potential third strand binding targets that should be protected. c, negative control third strand PsT-3, containing the same 6-nit duplex-forming hook and (T)_{4} linker as PsT-2, that is however followed by a scrambled sequence that should not form triplex.

**RESULTS**

**Experimental Plan**—Fig. 1, a and b, show the 38- and 35-base pair linear target fragments, D_{1}-D_{2} and D-1-D-2, respectively, of the β-globin gene sequence of human chromosome 11 used in this investigation. D-1-D-2 is a version of D_{1}-D_{2} shortened at the downstream end. Residue T_{11}, on strand D_{1}-I/D-1 (shown in bold) is the consequence of the A→T transversion responsible for sickle cell anemia. Residues 1–17 of strand D-2 (4–21 on D_{2}) and 12–19 on D-1/D_{1} comprise the 24-nit purine-rich third strand binding region that was targeted for triplex formation. The one T and three C residues within the purine-rich sequence of the D-2 binding domain significantly decrease the effectiveness of third strand binding to the target. Our original approach was a strand-switching third strand with an acridine intercalator added at the 5'-end (Fig. 1a). The triplex forming domain of PsT-1 was designed to bind to the D_{2} target segment in the pyrimidine/parallel motif, and then cross over to bind to the D_{1} segment in the GT/antiparallel motif. This design allows the psoralen moiety at the 3'-end of PsT-1 to be positioned directly opposite the mutant T_{11} residue of D_{2}. To enhance the association to this target, pr°U² was positioned opposite the three C-G inversions, G opposite the T-A inversion (15, 16), and me°C was substituted for C in the third strand opposite G-C pairs (17). Despite these various modifications, binding was not sufficiently enhanced (see below).

The strategy ultimately employed to achieve more effective third strand binding to the target in a linear duplex fragment...
is depicted in Fig. 1b. Additional binding energy was designed into the system by truncating the 3'-end of the D1-1 target strand (resulting in the D1-1 strand) to produce a 6-nt single-stranded “sticky” 5'-end of D-2. The 5'-end of PsT-1 (third strand) was then elongated in complementary fashion (via a linker of four T residues) to produce a duplex-forming hook to bind to the D-2 sticky end (Fig. 1b), resulting in third strand PsT-2. The combined strategy of using stronger binding modified residues, crossing-over of the third strand, and a duplex-forming hook proved successful in forming the structure in Fig. 1b. PsT-3, a scrambled sequence containing the 5'-duplex binding hook and 3'-psoralen, was used as a negative control (Fig. 1c).

**Third Strand Binding—Band-shift assays at 4 °C evaluated by native PAGE were used to assess third strand binding.** Fig. 2 shows that both PsT-2 and PsT-3 induce a band-shift of 32P-labeled target duplex D-1-D-2, whereas PsT-1, the hookless third strand, does not with D1-1-D2. This indicates that the binding energy between the sticky end of D-2 and the 6-nt hook of either PsT-2 or PsT-3 is sufficient to form a stable complex with apparent triplex stoichiometry under the electrophoretic conditions, whereas that between the triplex-forming domain alone and the duplex target is not. However, the results with PsT-2 and PsT-3 do not discriminate between true triplexes and complexes in which the third strand is merely bound by the hook. Note also that the mobility of the PsT-3-containing complex is somewhat lower, which may be a consequence of the “dangling” third strand making the complex much less compact than a (presumably) true triplex formed by PsT-2. Furthermore, electrophoretic analysis at 25 °C shows no band-shift for PsT-3, whereas PsT-2 does form a complex (data not shown). This is consistent with melting by that temperature of the hook from the complex that is not a triplex.

**DNase I protection experiments** (18) were performed to discriminate between triplexes, which should be relatively protected, and complexes formed merely by the third strand hook, which should be sensitive to the enzyme. Fig. 3a shows the results of denaturing PAGE analysis of such digests. Complexes formed with 10:1 (0.5 μM duplex, 5 μM PsT-2) and 100:1 (0.5 μM duplex, 50 μM PsT-2, data not shown) ratios of PsT-2: duplex display reduced sensitivity to DNase I along D-2 target segments B and C, but not along the unprotected segment A (cf. Fig. 1a). In contrast, complexes formed with similar ratios of the negative control strand PsT-3 do not display reduced DNase I sensitivity along any segment of D-2.

The gel from Fig. 3a was quantitated, and the photodensity of each D-2 segment was determined. These results, expressed in Table I as the fraction of the total strand cuts within each D-2 segment, confirm that complexes formed with PsT-2 display much reduced DNase I sensitivity along the D-2 target segments B and C, but not at unprotected segment A. This protection is very strong for segment C, but less evident for segment B (Table I), which contains the strand-switching triplex domain. Further, in Fig. 3b, where DNase I sensitivity is shown relative to that of naked duplex, the data clearly indicate protection along the D-2 target segment in the presence of PsT-2, but a lack of protection in the presence of PsT-3. Thus, triplex formation occurs only with PsT-2; so PsT-3 must be bound to duplex only by the hook.

**UV Irradiation of Complexes**—The complexes formed with PsT-1, PsT-2, and PsT-3 were UV-irradiated and the products analyzed by denaturing PAGE. Based upon the expected triplex structure, a psoralen on the 3'-end of PsT-1 or PsT-2 should principally form monoadducts (19) to residue T11 on the D1-1-D2-1 strand, resulting in covalent attachment of the third strand. The length of the linker (C16) between the psoralen moiety and the third strand makes possible additional monoadducts to Tn of D1-1-D2-1 and to various pyrimidine residues along D2-D2-2, the complementary duplex strand. Interstrand psoralen crosslinks (19) are also possible between pyrimidine residues of D1-1-D2-1 and D2-D2-2.

**Fig. 4** shows denaturing PAGE analysis of UV-irradiated complexes containing 32P-end-label on the D1-1-D2-1 strand that contained either 100 nM or 40 μM of the appropriate third...
strand. Photoproducts are observed in the presence of PsT-2, and also in the presence of the hookless PsT-1 at 40 μM; neither duplex alone nor any complex of PsT-3 and duplex produce higher molecular weight bands, notwithstanding the psoralen moiety tethered to the 3'-end of PsT-3 (Fig. 4).

Third strand binding affinity of PsT-1 and PsT-2 was evaluated by the dependence of photoproduct formation on third strand concentration. Varying concentrations of third strand were annealed to target duplex, irradiated, and analyzed by denaturing PAGE analysis (Fig. 5a). Bands were quantitated (Fig. 5b), giving apparent $K_d$ values of $<3$ nM for PsT-2 and ~3,000 nM for PsT-1. The 1,000-fold difference in $K_d$ values between PsT-1 and PsT-2 clearly demonstrates the advantage of the third strand with the duplex-forming hook, and all further work was performed using PsT-2.

Photoproduct Identification—To investigate the interaction of psoralen-linked third strand with the coding and noncoding target strands, irradiation experiments were performed using duplex with either $^{32}$P-D-1 or $^{32}$P-D-2. Triplexes with D-1 labeled show a major photoproduct and two minor ones, one slightly faster moving than the major one, and one much slower-moving. The D-2-labeled triplexes (Fig. 6a) also show one major photoproduct, but three faster moving minor products, and one much slower moving one. The slower moving band has the same mobility in both cases and it is also visible when PsT-2 is labeled (not shown). Hence, this slower moving photoproduct contains D1, D2, and PsT-2; it is therefore identified as a crosslink between PsT-2 and D-1-D-2. Based upon the sequence of the triplex, previous observations (17), the yield, and primer extension results described below, the major D-1-labeled photoproduct is identified as a monoadduct between PsT-2 and D-1 at residue T$_{11}$, whereas the faster running minor product is probably the monoadduct formed at residue T$_{9}$ of D-1. Similar considerations, including primer extension results (not shown), identify the major D-2-labeled photoproduct as a monoadduct between PsT-2 and residue T$_{22}$ of D-2 and the three faster moving minor products as monoadducts formed with residues C$_{21}$, C$_{22}$, and C$_{23}$, respectively.

Kinetics of Photoproduct Formation and Yields—The major D-1 monoadduct, which amounts to more than 50% of the total photoproduct, represents psoralen interaction with the mutated T nucleotide of the human sickle cell hemoglobin gene. As indicated in Fig. 6b, ~40% of the D-1 strand is converted to the major photoproduct by 5 min of irradiation. In contrast, the photocrosslinked product forms at much lower yield (≤3%) and does not level off over the timecourse studied, which is consistent with previous observations (5, 19). The kinetics of major photoproduct formation is similar for the D-1- and D-2-labeled complexes (Fig. 6b). The maximum yield is attained by 5 min of irradiation and levels off. The yield is significantly lower for the major D-2 monoadduct (15 versus 40%). This is consistent with the more distant location of the target residue in that strand and the less favorable orientation of the intercalated psoralen moiety (18).

Fig. 7 shows the dependence of photoproduct yield on pH. The yield decreases significantly as pH is increased from 5 to 7. Additional modifications of the third strand sequence that use C analogs that need not be protonated should improve binding at physiological pH (22, 23).

Sequenase Primer Extension—To confirm the photoaddition site of the major D-1 photoproduct, it was eluted from PAGE gel slices and used as a DNA template in primer extension

**Table 1**

| Structure | Segment |
|-----------|---------|
| A         | B       | C   | B + C |
| Duplex    | 0.5 μM  | 22.2| 40.5 | 37.3 | 77.8 |
| + PsT-2, 0.5 μM | 34.2 | 37.1 | 28.7 | 65.8 |
| + PsT-2, 5 μM | 55.6 | 29.1 | 15.3 | 44.4 |
| + PsT-2, 50 μM | 49.1 | 29.8 | 21.1 | 50.9 |
| + PsT-3, 5 μM | 24.6 | 35.6 | 38.8 | 75.4 |
| + PsT-3, 50 μM | 24.2 | 32.4 | 43.4 | 75.8 |

**Fig. 4. Photoproduct formation along D-1-labeled complexes.** Denaturing PAGE analysis of photoproduct formation. Complexes formed at 10 nM duplex and indicated third strand concentrations were UV-irradiated for 10 min at 4 °C. Note that no photoproduct is observed at either third strand concentration in the presence of the nontriplex-forming strand PsT-3, despite the psoralen tethered to its 3'-end. In contrast, strand PsT-2 forms photoproducts in similar yield at both concentrations. However, PsT-1 forms photoproducts only at very high (40 μM) concentration.

**Fig. 5. Binding affinity of third strands PsT-1 and PsT-2.** a, denaturing PAGE analysis of irradiated complexes formed with either 10 nM D$_{1}$-D$_{2}$-2 and increasing concentrations of PsT-1 (right panel) or 1 nM D-1-D-2 duplex and increasing concentrations of PsT-2 (left panel), using $^{32}$P-end-labeled D$_{1}$ and D-1 duplex strands, respectively. b, binding affinity of PsT-1 (•) and PsT-2 (△) determined from denaturing PAGE. Results are expressed as percent of labeled duplex converted to complex with PsT-1 or PsT-2. Higher yield for PsT-1 photoproducts could be due to higher duplex concentration.
reactions (20, 21). Fig. 8 shows these results along with a mixed ddNTP sequencing ladder obtained from nonirradiated D-1 control strands. It can be seen that synthesis continues only up to residue T11. Apparently, the polymerase is unable to synthesize past the PsT-2 photoattachment site. This observation identifies residue T11, the site of the A\textsuperscript{3}T\textsubscript{3}T\textsuperscript{z}A human sickle cell transversion, as the major site of psoralen photoaddition.

Similar observations on photoproducts formed with the D-2 target strand confirm the sites of photoattachment indicated above (data not shown).

Specific Third Strand Binding and Photoaddition to Plasmids Containing the Target—Band-shift assays (Fig. 9) indicate that whereas PsT-3, the scrambled third strand, does not bind to the plasmid with the \(\beta\)-globin sickle cell target, PsT-2, the third strand with the duplex-forming hook, does bind to it, though not to the vector plasmid without the target. Moreover, the binding is very much greater to the supercoiled than to open circular or linearized plasmid. These results suggest high promise for our overall approach.
DISCUSSION

With respect to our long-term goal, this study indicates that the efficiency of photochemical modification at the desired target site is relatively high; 40% of the duplex target is converted to monoadduct at the pyrimidine residue of the mutated base pair. Another 2% at this site are crosslinked, whereas a minor photoproduct on this strand accounts for ~8% of the duplex. The C₁₆ psoralen linker additionally permits substantial photoproduct formation of the opposite target strand. Model building suggests that shortening the linker should substantially reduce or even eliminate those reactions, in which case the yield and specificity of the desired psoralen photoaddition can be raised. In fact, preliminary experiments demonstrate that use of a C₉ psoralen linker markedly reduces photoproduct formation with the noncoding strand.

It would appear that early steps of a strategy for triplex-mediated repair of the sickle cell mutation are now in place. A strand has been designed with suitable affinity for third strand binding, which forms a psoralen monoadduct to the mutated base pair in high yield. DNA under superhelical stress unwinds binding, which forms a psoralen monoadduct to the mutated strand has been designed with suitable affinity for third strand mediation repair of the sickle cell mutation are now in place. A photoproduct formation of a third strand with a hook is capable of binding to a single-stranded patches no less accessible than the sticky end in the linear target. In fact, the preliminary results suggest that this third strand with the hook is capable of binding to a supercoiled plasmid containing the target sequence, indicative of a strand-invading mechanism (28). This opens the possibility of exploiting various DNA repair mechanisms that have been shown to effect site-specific base pair changes. For example, TA → AT substitutions in the supF gene have been triggered in vivo by third strand-directed psoralen photomodification of plasmids transfected into monkey COS-7 cells (6–8). Using the methodology developed, it is anticipated that the sickle cell mutation may be similarly repaired. In that event, acceptable levels of the correct phenotype might be achieved if psoralen monoadduct-triggered in vivo mutation efficiencies are sufficiently high.

Chimeric structures with linked duplex and triplex elements have been exploited for other purposes (24–28). In this study, the duplex-forming hook represents a novel approach for achieving effective third strand binding to a relatively poor DNA duplex target, coupled to a potential for strand invasion. Direct comparison of binding affinity to the same target (by photoproduct formation) of a third strand with a hook versus a hookless third strand demonstrates at least 1,000-fold difference in $K_d$ values. Moreover, the hookless third strand does not induce a band-shift on native PAGE even at high third strand concentrations (Fig. 2). This indicates that the complex is transient and is trapped only by formation of covalent photoproducts under UV-irradiation. In contrast, the addition of the duplex-forming hook to the third strand makes the complex stable even without irradiation. The approach developed in the present work should find wider applicability to other unfavorable target sequences.

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