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Testing of Thiol-Terminated Oligonucleotides as Potential DNA-Cleaving Reagents

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Abstract

In this study we have conducted experiments to determine if oligonucleotides containing terminal thiol groups can be used, in the presence of Cu^{+2} , to cut single- and double-stranded DNA molecules adjacent to the oligonucleotide binding site. The results of these experiments demonstrate that these modified oligonucleotides cleave DNA in the presence of Cu^{+2} , but the reaction could not be controlled sufficiently to attain site-selective cleavage or minimize degradation of the thiol terminated oligonucleotide.

Introduction

The field of molecular biology blossomed following the discovery and commercial introduction of restriction enzymes and several other proteins (polymerases, ligases, and other nucleases) that could be used to manipulate and synthesize DNA molecules. These molecular tools are now being used routinely throughout the world in all aspects of biological, medical, and forensic research.

Restriction enzymes, in particular, play an important role in molecular biology and genomic research by providing a mechanism for cutting DNA at specific sites designated by particular base sequences. This is an essential step required for cloning DNA molecules into vectors. It is also used routinely to identify and map DNA sequences. These proteins recognize a specific base sequence containing from four to seven bases, bind to the sequence, and cut both strands of the molecule near the recognition site. While more than 100 different restriction enzymes are commercially available, these enzymes recognize a relatively small number of different base sequences.

Because the number of different base sequences that are recognized by restriction enzymes is limited, investigators have begun to look for additional methods to selectively cleave DNA. Restriction enzymes that recognize larger, uncommon sequences in the genome have become important for genomic mapping. Several chemical agents, including Cu-phenanthroline [1,2] and complexes of Fe with bleomycin [3], methidiumpropyl EDTA [4], methylpyrrolicarboxamide EDTA [5], or porphyrins [6], have been examined as reagents for cleaving DNA. All appear to require the presence of oxygen and involve the formation of free radicals when the metal complex intercalates or binds to the DNA molecule. At least two have been shown to exhibit some degree of base specificity in their binding and cleavage sites. While 1,10 phenanthroline-Cu and EDTA-Fe have both been conjugated to oligonucleotides and shown to cut DNA [7,8,9], the preparation and purification of each of the oligonucleotide derivatives represents a significant undertaking.

Several years ago Reed and Douglas [10,11] demonstrated that free thiols in the presence of μM amounts of Cu^{+2} produced single stranded breaks in DNA. A wide range of thiols were found to be reactive, but only Cu^{+2} ions would catalyze the reaction. This discovery, and the recent advance in DNA synthetic capabilities that allow the routine production of oligonucleotides terminated with functional groups at the 3' or 5' ends of the molecules, provides a possible mechanism for developing a new class of reagents that could be tailored to recognize and bind to any DNA base sequence and insert a single or double

stranded DNA break adjacent to it. These reagents, oligonucleotides terminated with thiol groups, could be synthesized to order using existing automated DNA synthesizers. This would provide the investigator the ability to cut DNA at a single, specified spot anywhere in the genome. This technology, once available, could also be used to cut and retrieve very large segments of the genome located between any two known sequences (greatly facilitating genome mapping). It might eventually be adapted for use in gene inactivation (knockout) experiments.

Materials and Methods

Synthesis and Purification of Normal and Thiol-Terminated Oligonucleotides

Three oligonucleotides (30-, 42- and 72mers) were synthesized at the 0.2 micromole level using the ABI 394 synthesizer and standard beta-cyanoethyl phosphoramidites (ABI, Foster city, CA). The trityl group was retained on the 5'-terminus for subsequent reverse phase HPLC purification which was followed by cleavage of the group with 80% acetic acid and removal of the trityl group by a quick chromatographic (HPLC) step. The product was then lyophilized. Quantitation of the amount of oligonucleotide synthesized was based on the overall yield reported by the trityl monitor that exists as an accessory to this machine.

Two different methods were used to synthesize the 18 base thiol-terminated oligonucleotide (TTO) to be used in the cleavage reactions. The synthesis was performed as described for the oligonucleotides above except that on the last addition to the 5'-site, a 5'-thiol modifier C6 (Glenn Research, Sterling, VA) was used to produce a thiol-terminated oligo. The final oxidation step in this synthesis had to be carried out with 0.02M iodine solution to minimize oxidative cleavage of the trityl-S-linkage. This trityl group is not acid labile but is removed by reacting the oligo with aqueous silver nitrate solution for 30 minutes followed by a DTT solution which precipitated the silver-DTT complex. After centrifugation, the supernatant containing the 5'-thiol oligo was removed and lyophilized.

A second mode of synthesis used the 5'-thiol-modifier C6 S-S reagent (Glenn Research, Sterling, VA). Procedures for the addition were similar to the one described above with the exception that the trityl group was removed by 0.05M DTT added to the standard ammonium hydroxide deprotection step. This procedure removes the base protecting groups and cleaves the disulfide linkage to generate the 5'-thiol.

Assay System for Analysis of Cleavage Reactions

To test the ability of thiol-terminated oligonucleotides to make a single stranded break in DNA in the presence of Cu^{+2} , a 72 base oligonucleotide (Figure 1) was synthesized for use as a test DNA. A thiol-terminated 18 base oligonucleotide (TTO) complementary to a region of the 72-mer located 10 bases in from the 5' end of the sequence was synthesized for hybridization to the 72-mer. Each of the potential cleavage product sequences (18, 30, 42-mers) were also synthesized for use as standards. After annealing the TTO to the 72-mer, the DNA was incubated in the presence of copper sulfate (1-100 μM) at 4°, 21°, 37° and 55°.

37°, or 45°C for variable lengths of time. The complex was subsequently denatured and the size of the oligonucleotides remaining were determined by high performance liquid chromatography (HPLC) on a reversed-phase PRP column using an acetonitrile gradient in Triethanolamine acetate (TEAA).

As an alternative system, the samples were prepared for electrophoresis and run in polyacrylamide (PAGE) gels to monitor the size of the oligonucleotides remaining after treatment. Oligonucleotide samples for analysis were dissolved in 30% glycerol and TBE buffer (0.045M Tris-borate, 0.001M EDTA) and layered in wells of a 20% PAGE diluted from a 30% concentrated stock solution of acrylamide/bisacrylamide (29:1 w/w). The gels were run for two hours at 90 volts in TBE buffer, pH 8.0. After electrophoresis, the gel was removed from the gel holder, placed in a tray, and stained with a solution of ethidium bromide (10ug/100ml) for 30 minutes. The gel was photographed under short-wave UV light using an orange filter. In this system, the undigested oligo, the 30 mer and 42 mers were shown to separate as individual bands.

Cleavage Reaction Conditions

A variety of conditions were tested to determine whether or not the thiol-terminated oligonucleotide could be induced to form single stranded breaks in the oligonucleotide to which it hybridizes. Typical experiments were performed using a 72mer:18mer oligonucleotide molar ratio of 1:2. Aliquots of copper sulfate (1-20µl of 0.1M solution) were added to 50µl of the oligonucleotide and the mixture was allowed to react for varying lengths of time from 1 hr to overnight. The reaction was then stopped by adding excess EDTA to the mixture to chelate the Cu⁺² and resolubilize the DNA. The concentration of copper used in the mix was varied from 1-100µM. In an effort to limit the cleavage reaction, experiments were also conducted with reaction mixtures containing different concentrations of glycerol as a free radical scavenger. Reactions were usually performed at 37°C, but experiments were also conducted at room temperature (~21°C) and 4°C with reaction times from 30 min to overnight.

Results and Discussion

The hypothesis behind our proposed method for using thiol-terminated oligonucleotides (TTO's) to cleave DNA at specific sequences is based on the known fact that free thiols, in the presence of Cu⁺² and oxygen, will generate free radicals that break the phosphodiester backbone of DNA. In the case of oligonucleotides modified to contain a Cu-phenanthroline moiety, the breakage of the DNA backbone was found to occur at several sites over an area (several bases) corresponding to the maximum radius of diffusion of the free radical (Figure 1). Using thiol terminated oligonucleotides, which are much easier to synthesize than the Cu-phenanthroline derivatives, we have hypothesized that a similar cleavage mechanism should occur.

To test this hypothesis, we have synthesized TTO's by two different methods, annealed them to a synthetic 72 base DNA sequence, and treated the complex with dilute copper solutions to attempt to effect site specific cleavage of DNA. The initial oligonucleotide to be tested was an 18 base sequence containing a free thiol group attached to the 5' end of the sequence using a C6

linker. The base sequence used was complementary to a region of the 72mer located 10 bases in from its 5' end (Figure 2). The test system was designed so cleavage could be detected by the loss of the 72mer oligo and the appearance of oligos of intermediate size containing approximately 18, 30± and 42± bases. If the cleavage occurs immediately adjacent to the 5' end of the bound TTO, the 72mer should be cleaved into two fragments containing approximately 30 and 42 bases. Both these fragments are different enough in size to be distinguished from the TTO (18 bases) and the undegraded 72mer. The actual size distribution can be used to assess the specificity of the cleavage reaction and the variability in cut site. The size of the DNA fragments present after treatment with Cu+2 are determined by HPLC and gel electrophoresis.

Based on preliminary studies we had conducted which suggested that the TTO's might be unstable, two different methods were used to synthesize the derivatized oligonucleotides. One involved the synthesis of a thiol derivative blocked with a trityl group. The S-trityl bond could not be cleaved with acid, and additional steps were required to eliminate the trityl group by displacing it with silver. Immediately prior to use, the silver was removed by treatment with DTT. The second method involved synthesizing a disulfide derivative of the TTO. TTO's synthesized by this method were easier to prepare for use because they only needed to be reduced by DTT treatment immediately prior to use. Both derivatives appeared to be reasonably stable.

Two different assay systems were used to assess the extent of cleavage induced by Cu+2 and the size of the resulting fragments. An HPLC system was developed and optimized to separate the expected fragments and tested using synthetic 30, 42 and 72 base oligonucleotides corresponding to the theoretical cleavage fragments (Figure 3). All three fragments could be easily resolved. Only the thiol-terminated 18 mer did not resolve as a separate peak because the thiol derivative of the 18mer co-eluted with the 72mer. The size of the fragments could also be assessed by electrophoresis in polyacrylamide gels.

TTO's synthesized by both methods were tested for their ability to cleave the 72mer in the presence of Cu+2 ions. In both cases, the instability of the TTO's once they were deblocked proved to be a significant problem. This affected not only on their ability to cleave DNA to which the molecules were bound, but it also had a significant impact on the lifetime (shelf-life) of the oligonucleotide itself. As shown in Figure 4, at least 90% of the TTO degrades by 24 hrs after deprotection of the thiol group. The blocked derivatives were found to be completely stable. Consequently, the TTO's had to be freshly deblocked just prior to use to minimize their degradation.

In contrast to previous methods that have been used to cleave DNA, such as the Cu-phenanthroline method, our approach involved sequestering the thiol near the site to be cleaved by attaching it to the 3' end of the oligonucleotide. The Cu+2 required to catalyze the formation of free radicals was then introduced into the system in the buffer. Binding of the 18 base TTO to the 72mer was confirmed by gel electrophoresis as a slight shift of the 72mer band to a lower mobility (Figure 5). A range in Cu+2 concentrations (1-100µM) was tested to effect cleavage of the complex. Unfortunately, even the most dilute Cu+2 concentrations precipitated the DNA, and the treated DNA in these samples had to be resolubilized prior to electrophoresis using EDTA. We did attempt to use the chelating agent EDTA to prevent this Cu+2 induced DNA precipitation and limit

non-specific degradation. But the results obtained with EDTA were not different than those obtained without it.

The results of these experiments showed that the TTO's do cleave DNA in the presence of Cu^{+2} , but the cleavage reaction is not limited to only the long test sequence. Whenever conditions were achieved that resulted in cleavage, both the 72-mer and the TTO were completely degraded (Figure 6). Attempts to modulate or minimize the extent of this degradation by incorporating increasing concentrations of free-radical scavengers into the reaction buffer (glycerol) were also unsuccessful. To date, we have not identified conditions that can be used to limit the reaction sufficiently to attain site-selective cleavage or minimize TTO degradation. The fact that the reaction occurs is promising, but additional work will be required to eliminate non-specific cleavage. This may require limiting the amount of free Cu^{+2} that is available to the DNA (e.g. using an equimolar ion-exchange reaction).

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Figure Legends

Figure 1. Reaction scheme for cleavage of DNA by thiol-terminated oligonucleotides. The upper panel shows the proposed mechanism for other metal tagged oligonucleotides. The lower panel show the method using TTO's and free Cu^{+2} in the buffer.

Figure 2. DNA sequence of the 72 base test oligonucleotide and the 18 base thiol-terminated oligonucleotide used in this study.

Figure 3. Separation pattern for synthetic oligonucleotide standards chromatographed on a reversed-phase PRP column using an acetonitrile gradient in TEAA as described in the Materials and Methods section.

Figure 4. Stability of the 18 base TTO. Upper panel shows HPLC chromatograph of the freshly prepared TTO. Lower panel show the same amount of sample injected after 24 hrs storage. Less than 10% of the TTO remained undegraded after 24 hrs.

Figure 5. Polyacrylamide gel showing the standard oligonucleotides used in the experiment (lanes 1-3, 6-8) and the 18mer annealed to the 72mer prior to Cu^{+2} addition (lane 4).

Figure 6. Cleavage of the 72mer test sequence following complexation with the TTO and treatment with Cu^{+2} . A. HPLC chromatograph of the 72mer alone. B. HPLC chromatograph of the 72mer complexed with the thiol-terminated oligonucleotide. C. HPLC chromatograph of the 72mer-TTO complex after treatment with 45mM Cu^{+2} . The complex is completely degraded, as revealed by the absence of a peak at 21 min corresponding to the 72mer-TTO complex.

REACTION SCHEME:

Previous

Chelated Cu^{+2} or Fe^{+2}
 R-SH
 O_2

Cu-phenanthroline*
 Fe-bleomycin
 Fe-methidiumpropyl EDTA*
 Fe-methylpyrrolecarboxamide EDTA
 Fe-porphyrin

Proposed

Thiol-terminated oligo
 Cu^{+2}
 O_2

Expected Point of Cleavage

5'-GTCCACGCGATCAAGTATAGCTCGACGCTCAGCTGTTAACGGGCCCCGGGTACCGCGATATCGATCTTTAAAG-3'

3'-TGCAGTCGACAATTGCC-SH

18 base TTO

72 base test oligonucleotide

F142

Fig. 3

The graph displays a signal intensity profile. The x-axis is labeled from 0.000 to 22.000. The signal begins with a very sharp, high-intensity peak at approximately 0.500. It then drops to a baseline level. There are several smaller peaks: one around 4.000, a small one around 8.000, and a more prominent one around 18.000. The signal returns to the baseline after the peak at 18.000.

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