

# Triplex-mediated cleavage of DNA by 1,10-phenanthroline-linked 2'-O-methyl RNA

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Received 24 February 1996

**Abstract** We have previously reported that 2'-O-methyl RNAs are efficient probes for duplex DNA. Here we describe the design, synthesis, and DNA cleaving activity of 1,10-phenanthroline (OP)-linked 2'-O-methyl RNA (OP-m). Although a local triple helix was formed, both with OP-m and a control OP-linked DNA at the target sequence of the duplex DNA, the promoter region of the human thrombomodulin gene, the cleavage efficiencies on both strands were not proportional when OP-m was used as a cleavage agent. These results may reflect the structural differences of the respective triple helices and the duplex-triplex junction, formed from the two types of triplex-forming oligonucleotides, 2'-O-methyl RNA and DNA. Since the OP-ms were found to work as preferential purine-strand cutters for duplex DNA, they would be useful as unique tools for genome analysis.

**Key words:** 2'-O-Methyl RNA; Triple helix; 1,10-Phenanthroline; Human thrombomodulin gene; Chemical cleavage

## 1. Introduction

Homopyrimidine oligonucleotides recognize homopurine-homopyrimidine double-helical DNA through triple helix formation [1]. The specificity is imparted by Hoogsteen bond formation in the major groove of the DNA. To develop an effective probe for duplex DNA, several modifications of the bases or the phosphates of the triplex forming oligonucleotides (TFOs) have been made. We have reported that 2'-O-methyl RNAs are novel probes for duplex DNA, and that the triple helix structures formed from double-stranded DNA and 2'-O-methyl pyrimidine oligonucleotides show the highest thermal stability among the various 2'-substituted oligonucleotides: 2'-deoxy, 2'-fluoro, and RNA [2]. A detailed thermal melting study using 2'-O-methyl RNAs, by Wang and Kool [3], supports our findings. Further investigations of 5-methyl analogs of 2'-O-methyl RNAs showed that a TFO containing 5-methyl-2'-O-methyluridine formed a more stable triple helix with the target DNA than the 5-unmodified parent probe [4]. In this study we converted 2'-O-methyl RNAs with a thiophosphate at the 5' end into chemical nucleases, which recognize the homopurine-homopyrimidine clusters in the

promoter region of the human thrombomodulin (TM) gene [5,6]. The DNA cleaving moiety was introduced into the TFOs by reaction with a thiol-specific agent bearing 1,10-phenanthroline (OP) [7].

## 2. Materials and methods

### 2.1. Chemicals

Oligonucleotides were synthesized on an Applied Biosystems (ABI) 394 DNA/RNA synthesizer using the standard phosphoramidite method [8], with commercially available reagents (ABI for DNA) and 3'-(2-cyanoethyl)-N,N-diisopropylphosphoramidite derivatives of 2'-O-methyl ribonucleosides [9]. The phosphoramidite derivative of N<sup>4</sup>-benzoyl-5-methyl-2'-deoxycytidine was prepared as described previously [7]. Oligonucleotides were purified by reverse phase HPLC using an Inertsil ODS-2 column (10 mm×250 mm, GL Science Inc.). Further purification was done by anion exchange HPLC using a TSK gel DEAE-2SW column (4.6 mm×250 mm, Tosoh Co.). The synthesis and purification of the OP-linked oligonucleotides (OP-oligos) were conducted based on the reported procedures [7].

### 2.2. Preparation of 5' end-labeled double-stranded 50-mer DNAs

Oligodeoxynucleotides (ODNs) corresponding to nucleotides 296–345 of the human TM promoter [5] were chemically synthesized and purified: d(CCAGGCACTTCCTTCCTTCCCGAACGTCCAGGAGGGAGGGCCGGGCA) (coding strand) and its complementary strand (noncoding strand). Both strands were 5' end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase (Takara Shuzo). The labeled strands were purified with a NENSORB 20 column (DuPont), and were annealed with the unlabeled complementary strand by heating at 80°C for 10 min and then slowly cooling to room temperature.

### 2.3. Cleavage of 50-mer double-stranded DNAs

A 4- $\mu$ l solution of the target 50-mer duplex DNA (0.1 pmol) and the OP-oligo (10 pmol), in a buffer containing 12.5 mM Tris-acetate (pH 7.0), 125 mM NaCl, and 1.25 mM spermine-4HCl, was heated at 70°C for 10 min and slowly cooled to 4°C for triple helix formation. 10  $\mu$ M copper sulfate (0.5  $\mu$ l) was added and the solution was pre-incubated at 37°C for 1 h. The cleavage reaction was initiated by adding 50 mM  $\beta$ -mercaptopropionic acid (MPA) (0.5  $\mu$ l). The solutions of copper ion and MPA were freshly prepared in ultra-pure water, purified by a Milli-Q system (Millipore) and by a TORAY ion exchange fiber (TORAY PURE LV-08). The reaction mixture was incubated at 37°C for 20 h. The cleavage reaction was stopped by adding 100  $\mu$ M neocuproine in ethanol (1  $\mu$ l). This solution was mixed (1:2, v/v) with 80% deionized formamide containing 0.1% xylene cyanol and 0.1% bromophenol blue. The samples were heated at 80°C for 10 min and chilled in ice for denaturing. They were then loaded onto a 15% polyacrylamide gel (19:1, acrylamide/bisacrylamide, 40 cm long) containing 7 M urea. The gel was run at constant voltage (1800 V). The gel was dried and exposed to an imaging plate. The radioactivity was analyzed by a bioimaging analyzer, FUJI BAS2000.

### 2.4. Preparation of labeled 213-bp fragments from the TM gene

The *FokI*/BssHII fragment (205 bp) of the TM gene [5] was isolated from a 2% Nusieve agarose gel (FMC Bioproducts) by electro-elution. The DNA was phenol extracted and ethanol precipitated. The coding and noncoding strands were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP and [ $\alpha$ -<sup>32</sup>P]dTTP, respectively, in the presence of Klenow enzyme (Boehringer-Mannheim). The labeled DNA fragment was purified on an 8%

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**Abbreviations:** A, 2'-deoxyadenosine; G, 2'-deoxyguanosine; C, 2'-deoxycytidine; T, thymidine; M, 5-methyl-2'-deoxycytidine; Um, 2'-O-methyluridine; Cm, 2'-O-methylcytidine; TFO, triple helix forming oligonucleotide; TM, thrombomodulin; OP, 1,10-phenanthroline; OP-oligo, OP-linked oligonucleotide; ODN, oligodeoxynucleotide; MPA,  $\beta$ -mercaptopropionic acid; OP-m, OP-linked 2'-O-methyl RNA; OP-d, OP-linked oligodeoxynucleotide.

non-denaturing gel. The isolated DNA was desalted by passage through a Nick column (Pharmacia), and was eluted with ultra-pure water.

### 2.5. Cleavage of 213-bp fragments by OP-TFOs via triple helix formation

For triple helix formation, a 4- $\mu$ l solution of labeled DNA ( $\sim 0.1$  pmol) and OP-TFO (10 pmol), in a buffer containing 12.5 mM Tris-acetate (pH 7.0), 125 mM NaCl, 1.25 mM spermine-4HCl, and carrier tRNA (0.5  $\mu$ g), was incubated at 37°C for 12 h. 50  $\mu$ M copper sulfate (0.5  $\mu$ l) was added, and the solution was further incubated at 37°C for 1 h. The cleavage reaction was initiated by adding 100 mM MPA (0.5  $\mu$ l). After 20 h at 37°C, the reaction was stopped by freezing with liquid nitrogen, and was then lyophilized. The DNA was dissolved in 5  $\mu$ l of loading dye containing 80% formamide, and was heat treated for denaturing. The cleavage reactions were analyzed on a 5% polyacrylamide gel containing 7 M urea.

## 3. Results

### 3.1. Design of the OP-linked 2'-O-methyl RNA directed toward the promoter region of the human TM gene via triple helix formation

To test the triple helix formation and the cleavage efficiency of an OP-linked 2'-O-methyl RNA (OP-m) on a target site, the promoter region of the human TM gene was chosen. According to the DNA sequences [5], two adjacent, possible triple helix forming sites, with different base compositions, are located in this region (Fig. 1). Triplex site 1 contains runs of pyrimidines on the coding strand and is a T-rich sequence (67% in 12 bp; d-TTCCTTCCTTTT, defined as the Y-site). A cluster of purines on the same strand is located 12 bp downstream, and has a G-rich character (75% in 12 bp; d-AGGGAGGGAGGG, defined as the R-site). These triplex sites are included in the regulatory region of the promoter, which is called region I [5]. We designed the two OP-ms (12-mers), which were expected to cleave the double-stranded DNAs individually at the target sites: TMY-m for the Y-site and TMR-m for the R-site (Fig. 2). For the control experiments, two OP-linked oligodeoxynucleotides (OP-ds) with different backbones were also designed: TMY-d and TMR-d (Fig. 2). The synthesis and purification of the OP derivatives of the 5'-thiophosphorylated oligonucleotides were performed based on the reported procedure [7].

### 3.2. Cleavage of $^{32}$ P-labeled 50-mer duplex DNAs

Synthetic 50-mer DNAs corresponding to the sequence from numbers 296 to 345 were prepared (Fig. 1). The coding

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181                               Fok I
TCCCTGCGCCAGGGCAGGGTTTACTCATCCGGCGAGGTG

ATCCCATGCGCAGGGCGGGCGCAAGGGCGGCCAGAGAAC

                                296
CCAGCAATCCGAGTATGCGGCATCAGCCCTTCCCACCAAGG

Triplex site 1 (Y-site)           Triplex site 2 (R-site)
CACTTCCTTCCTTTTCCCGAACGTCCAGGGAGGGAGGGCC

345
GGGCACCTTATAAACTCGAGCCCTGGCCGATCCGCATGTCA

                                BssH II
GAGGCTGCCTCGCAGGGGCTGCGCGCACGGCAAGAAGTGT

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Fig. 1. Partial nucleic acid sequence of the human thrombomodulin gene promoter, containing two possible triplex sites. The coding strand is indicated.

### 1. OP-oligonucleotides for Y-site

TMY-m: OP-m(UUUUCCUCCU)(dT)

TMY-d: OP-d(TTTTMMTTMMTT)

### 2. OP-oligonucleotides for R-site

TMR-m: OP-m(UCCCUCUCCU)(dC)

TMR-d: OP-d(TMMMTMMMTMMC)

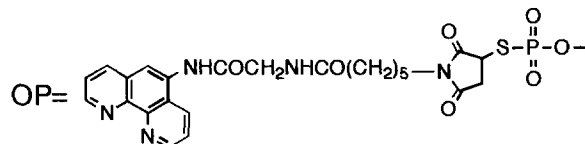


Fig. 2. Sequences of OP-oligonucleotides.

and noncoding strands were  $^{32}$ P-labeled and were each tested for chemical cleavage. A large excess (100 equivalents) of the OP-oligo was included in the buffer (pH 7.0), containing Cu(II) and a reducing agent (MPA). After a 20-h incubation at 37°C, the cleavage reactions were analyzed on a 15% denaturing polyacrylamide gel. Specific DNA cleavages of both targeted strands were observed in all reactions, and the efficiencies are summarized in Fig. 3. When TMY-m was used for targeting the Y-site, the thymine and protonated cytosine bases imparted hydrogen bonding to the purines on the non-coding strand, through Hoogsteen base pairing in the major groove. The bound oligonucleotides are located in a parallel orientation toward the purine strand [1], and the OP moiety is localized at the 3' end of the Y-site. The consequent double-stranded cleavage is considered to be caused by an intercalation of the OP moiety from the major groove at the duplex-triplex junction: the chelating center for the copper ion is located in the minor groove of the target DNA [10]. As shown in Fig. 3a, 74% of the cleavage was obtained on the labeled noncoding strand, which directly base paired with the added TMY-m. On the other hand, only 7% cleavage was observed on the complementary coding strand. Similar results were obtained when another triplex site was targeted using TMR-m (Fig. 3b). At the R-site, TMR-m binds to a purine-rich strand, and the resulting cleavage efficiency was high on the bound coding strand (53%), as compared with the noncoding strand (13%). Interestingly, these results are characteristic for OP-m and not for OP-d (control). Using TMY-d, both the coding and noncoding strands were cleaved with similar efficiency: 56% on the noncoding and 38% on the coding strand. In addition, when the R-site was targeted with TMR-d, almost equal efficiency was obtained on both strands: 45% on the coding and 51% on the noncoding strand.

### 3.3. Cleavage of the 213-bp restriction fragment

As it was shown that the OP-oligos with a 2'-O-methyl RNA as a recognition moiety have the ability to cleave the target sites of double-stranded DNA, we next applied these reagents to the site-specific cleavage of a 213-bp restriction fragment and tested the cleavage efficiency. Plasmids containing a TM promoter sequence [5] were digested with *FokI* and *BssHII*, and the desired DNA fragment was isolated. Selective labeling of either the coding or noncoding strand was per-

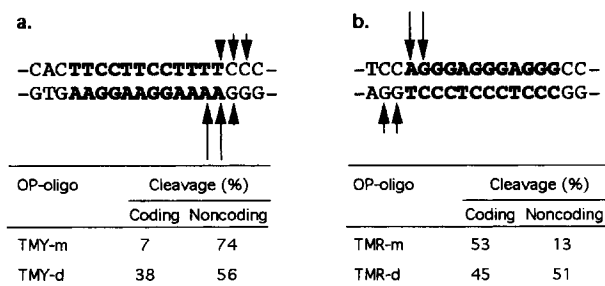


Fig. 3. Double-stranded cleavage at the two triplex sites of the synthetic deoxy 50-mer by OP-oligonucleotide conjugates: Y-site (a) and R-site (b). Arrows indicate the cleavage sites and the relative intensities of the reaction when OP-ms were used. DNA regions for the triple helix formation are indicated in bold.

formed by a fill-in reaction with the Klenow enzyme. In the presence of [ $\alpha$ - $^{32}$ P]dCTP, the coding strand was labeled at the *Bss*HII-cleaved site. In the same manner, the noncoding strand was specifically labeled with [ $\alpha$ - $^{32}$ P]dTTP at the *Fok*I-cleaved site.

A DNA fragment, labeled on either the coding or the noncoding strand, was incubated with a 100 molar excess of OP-m, in a buffer (pH 7.0) containing spermidine at 37°C, for triplex formation (12 h). The cleavage reaction was initiated by adding copper ion and MPA, as described above for the 50-mer cleavage. The cleavage products were analyzed on a denaturing polyacrylamide gel containing 7 M urea, as shown in Fig. 4. The cleavage sites were determined by the chemical sequencing of G+A ladders. Specific cleavage of the targeted purine strand was observed at the same DNA sequences as shown in Fig. 3 by using both OP-m (lanes 1 and 3) and OP-d (lanes 2 and 4). Although the overall cleavage efficiency was lower for OP-m (lane 1: 59% and lane 3: 38%), as compared to the OP-d (lane 2: 83% and lane 4: 60%), both reagents were found to act as site-specific chemical nucleases.

#### 4. Discussion

ODNs that bind or react sequence-specifically to double-stranded DNA have attracted much attention as biochemical tools. For example, TFOs can be used as potential chemotherapeutic agents for controlling gene expression at the level of transcription [11,12], and OP-oligos can be employed for chromosome mapping and the analysis of eukaryotic transcription units [13]. We describe here the design, synthesis, and DNA cleaving activity of OP-m. Although a local triple helix was formed by both OP-d and OP-m at the target DNA sequence, the cleavage efficiencies on both strands of the duplex were not proportional when the OP-ms were used as cleavage agents. These results may reflect the structural differences of the respective triple helices and the duplex-triplex junctions, formed by the two types of TFOs, 2'-*O*-methyl RNA and DNA. The structural alteration would change the geometry of the inserted OP-copper to some extent, and would affect the reactivity of the hydroxyl radicals generated in situ by the reduction of Cu(II) in the minor groove of the DNA [14]. Previous studies indicated that the triple helices of duplex DNA and TFO with different sugars have different migrations in non-denaturing polyacrylamide gels [2,15]. In addition, an affinity cleavage analysis, using TFOs with EDTA-Fe as a DNA cleavage agent, showed that the helical

geometry of the triplex can vary, depending on the backbone composition [16]. We assume that the triple-helical structure containing 2'-*O*-methyl RNA, as a TFO, has more A-like structure as compared to the DNA triple helix, because of the structural conservatism of the RNA [17]. Based on our results, it is possible to design an OP-m single strand-specific chemical nuclease by choosing a different linker group [18] to change the geometry of the OP insertion into the DNA. A promising application of the OP-ms for genome technology is their use to detect DNA regions that contain the adjacent clusters of homopurines and homopyrimidines in the 5' to 3' direction on the same DNA strand; e.g. such a DNA sequence is found in exon 5 of the hamster adenine phosphoribosyltransferase gene [19]. Since OP-ms were found to work as preferential purine strand cutters for duplex DNA, long stretches of 5'-protruding ends will be obtained by using two OP-ms. A subsequent fill-in reaction with labeled nucleotides and DNA polymerase (exonuclease-free) will give a strong signal on the genomic DNA. On the other hand, a derivative of a DNA-2'-*O*-methyl RNA chimera [20], in which

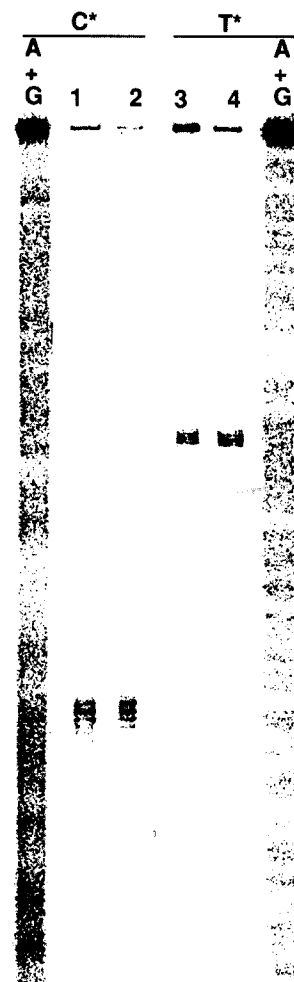


Fig. 4. Site-specific cleavage of the *Fok*I/*Bss*HII fragment of the human TM gene promoter by TMR-m (lane 1), TMR-d (lane 2), TMY-m (lane 3), and THY-d (lane 4) via oligonucleotide-directed triple helix formation. The fragment was labeled with either [ $\alpha$ - $^{32}$ P]dCTP at the *Bss*HII-cleaved site (C\*) or [ $\alpha$ - $^{32}$ P]dTTP at the *Fok*I-cleaved site (T\*). The A+G lane indicates the products of the Maxam-Gilbert chemical sequencing reaction.

the 5' portion of the OP-m is replaced with a few deoxynucleotides, may cleave both strands of DNA with equal efficiency. Structural studies, e.g. NMR studies or X-ray single crystal analyses, will be useful to determine the conformational differences between the two types of triplexes. The TFOs used in this study required cytosine protonations. Our data clearly indicate that the triplexes were sufficiently stable during the cleavage reaction at a neutral pH (7.0) in the presence of 1 mM spermidine. Although the overall cleavage efficiency of OP-m was almost equal to or less than the deoxy counterparts, the thermal stabilities of these OP-ms were higher than the OP-ds, as indicated by the *T<sub>m</sub>* measurements (TMY-m, *T<sub>m</sub>* = 56.6°C; TMY-d, 52.4°C; TMR-m, 69.2°C; TMR-d, 53.0°C, at pH 5.0 when the 50-mer DNA was used as a target). Since 2'-*O*-methyl RNA is considered to have several advantages over DNA for the formation of a pyrimidine+purine-pyrimidine-type triplex, including higher binding affinity for duplex DNA [2] even at low salt concentrations [4], mismatch discrimination ability [2], and nuclease resistance [21], OP-ms can be useful biochemical tools for the cleavage of large DNAs.

**Acknowledgements:** This research was supported by a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan. The authors would like to thank Dr. Robert D. Rosenberg (MIT, Cambridge, MA) for providing the human thrombomodulin gene for this study.

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