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Oligo(2′-*O*-methyl)ribonucleotides

Effective probes for duplex DNA

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To find novel probes for duplex DNA, we prepared four types of triplexes containing a homopurine-homopyrimidine 15-mer duplex DNA, and examined their thermal stabilities (T_m values). The single strands used for triplex formation were a DNA 15-mer having a defined C-T mixed sequence, and its sugar-modified analogs, namely 2′-fluoro DNA, RNA, and 2′-*O*-methyl RNA. The 2′-*O*-methyl RNA and the RNA-containing triplexes were similar in their enhanced stabilities at pH 6.1 and, amongst the four triplexes, the 2′-*O*-methyl was the most stable at pH 5.0. Furthermore, an experiment using a 34-mer duplex DNA suggested that the 2′-*O*-methyl RNA-triplex was destabilized, mostly as a result of the incorporation of a mismatched triplet, as compared to the DNA triplex counterpart. Thus, 2′-*O*-methyl RNA can serve as an effective probe for duplex DNA.

2′-*O*-Methyl RNA probe; Triplex formation; Sugar modification; Duplex DNA; T_m value; Native polyacrylamide gel electrophoresis

1. INTRODUCTION

Homopyrimidine oligonucleotides bind parallel to homopurine sequences via Hoogsteen bond formation (A-T and G·CH+ pairings) in the major groove of a homopurine–homopyrimidine duplex DNA to form a triple helix [1,2]. There have been reports on several approaches to modify the introduced third strand in order to obtain an efficient biochemical tool. For example, modified base residues, such as 5-methylcytosine [3,4], 5-bromouracil [4], and pseudoisocytosine [5] have been incorporated into the strand. Attempts to neutralize the negative charge of the phosphate backbone have also been done [6–8]. Here we report an alternative strategy to modify the DNA sugar residues and the use of oligo(2′-*O*-methyl ribonucleotides) for recognition of the DNA duplex.

We have previously demonstrated that oligo(2′-*O*-methylribo-nucleotides) form stable duplexes with RNA fragments [9] and have reported their use as biochemical tools [10]. Solution and X-ray studies showed that 2′-*O*-methyl modification of single- or double-stranded RNAs does not cause any significant conformational change [11–13]. On the other hand, NMR studies [14,15] and polynucleotide fiber diffraction analyses [16] of DNA triplex show that the third strand has an RNA-like, C3′-*endo* sugar puckering, although a DNA single strand alone usually adopts a C2′-*endo* conformation. From these findings, we envisioned that the 2′-*O*-methyl RNA may bind more effectively and tightly to a DNA duplex than a third DNA strand if the 2′-*O*-methyl RNA had a C3′-*endo* conformation and the methoxy groups enhanced the rigidity of the triple-stranded structure. Although studies on the formation and stability of RNA-containing triplexes were reported earlier [17,18], there is no precedent for the use of RNA oligomers with defined sequences.

It has been proposed that either the 2′-*endo* or the 3′-*endo* sugar conformation of the oligonucleotides should be characterized by a 2′-substituent [19]. In order to compare and evaluate the thermal stability of a triplex containing a 2′-*O*-methyl RNA, we chose three oligomer counterparts having different sugar residues, namely 2′-deoxyribose (2′-endo), 2′-deoxy-2′-fluororibose (3′-endo), and ribose (3′-endo).

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2. MATERIALS AND METHODS

Oligonucleotides were synthesized on an Applied Biosystems (ABI) 394 DNA/RNA synthesizer using the standard phosphoramidite method [20] with commercially available reagents (ABI for DNA and MilliGen Biosearch for RNA) and 3′-(2-cyanoethyl)*N*,*N*-diisopropylphosphoramidite derivatives of 2′-*O*-methyl ribonucleosides [21]. 2′-Deoxy-2′-fluoro pyrimidine nucleosides and their 3′-phosphoramidite derivatives were prepared based on the reported procedures [22, 23]. We used a Um support for synthesis of oligo(2′-deoxy-2′-fluoro nucleotides). Products were purified by reverse-phase and anion-exchange chromatography and were finally passed through columns of AG 50W-X2 (pyridinium form followed by sodium form), and Chelex 100 (Bio-Rad). For gel electrophoresis, the 15-mer homopurine strand (dPu) and the 2′-*O*-methyl RNA (m15) were 5′-end-labeled with T4 polynucleotide kinase and ^{32}P-γATP.

2.1. *Thermal denaturation profiles*

UV melting studies were performed in a buffer containing 10 mM sodium acetate (pH 5.0), 0.1 M NaCl, and 1 mM EDTA or 10 mM sodium phosphate (pH 6.1), 0.1 M NaCl, and 1 mM EDTA. The oligonucleotide concentration was 1 μM as a single strand. Thermal denaturing curves were recorded at 260 nm on a Gilford Response II UV-VIS Spectrophotometer using the temperature programming. The cell path length was 1 cm. The mixture of duplex and single strands was first heated at 50°C for 10 min, then slowly cooled to 5°C. The temperature was then increased to 80°C at a rate of 0.4°C/min. The cuvette-holding chamber was flushed with N_2 gas for the duration of the run. Thermal denaturation profiles were evaluated by identifying the temperatures (T_m values) at which $d(A_{260})/d(T°C)$ vs. T(°C) plots (first derivatives plots) reached their maxima.

2.2. *Gel electrophoresis*

For gel retardation assays of the triplexes, a non-denaturing 15% polyacrylamide gel (29:1 acrylamide/bisacrylamide) containing 40 mM tris-acetate (pH 5.4) and 1 mM EDTA was prepared and run at 20°C using the same buffer. Each strand concentration was 2.5 μM. Duplex and triplexes containing labeled dPu or m15 were heated at 50°C for 10 min then slowly cooled to 4°C. The samples were then loaded in 15% Ficoll (Pharmacia type 400), and Bromophenol blue dye (indicated as BPB in Fig. 2) was used as a marker.

Table I

Sequence of the targeted duplex and the second pyrimidine strands in the triple-helix, and its binding orientation

Duplex	dPu	5'GGGAGGGAAGAGAAA3'
	dPy	3'CCCTCCCTTCTCTTT5'
Triplex		
2nd pyrimidine		5'NNNNNNNNNNNNNNN3'
	dPu	5'GGGAGGGAAGAGAAA3'
	dPy	3'CCCTCCCTTCTCTTT5'
Triplex		
2nd pyrimidine strands		
D15	d15	5'CCCTCCCTTCTCTTT3'
F15	f15	5'CfCfCfCfUfCfCfCfUfUfUfCfUfCfU-fUfUm3'
R15	r15	5'r(CCCUCCCUUCUCUUU)3'
M15	M15	5'CmCmCmUmCmCmCmUmUmCmUm-CmUmUmUm3'

Cfl=2'-fluorocytidine, Ufl=2'-fluorouridine, Cm=2'-*O*-methylcytidine, Um=2'-*O*-methyluridine.

3. RESULTS AND DISCUSSION

We chose the same 15-mer mixed sequences as previously used to examine DNA triplex stability [24]. The sequences and the polarities of the duplex and triplexes are listed in Table I.

At pH 6.1, the target duplex DNA, consisting of a homopurine 15-mer and its complementary homopyrimidine 15-mer, had a T_m of 51.7°C (Fig. 1a). As shown in Fig. 1a–d, with increasing temperature, the UV melting profiles for D15, F15, R15, and M15 gave a biphasic dissociation that was typical of a triple helix. Since the second transitions had the same T_m (51.4 ± 0.4°C) as in the case of the duplex alone (Fig. 1a), it was easily identified as the transition corresponding to the denaturation of the Watson–Crick hydrogen bonds. Hence, the first, lower T_m values were assumed to be the melting of the third strand from the underlying duplex. The first T_m (36.8°C) of M15 was higher than that (28.3°C) of D15, as expected, and was almost the same as R15 at pH 6.1 (Table II).

In a more acidic solution (pH 5.0), which favored the protonation of cytosines, all the thermal denaturation profiles showed monophasic helix–coil transitions which were similar to that observed for poly d(T-C)·d(G-A)·(U-CH^+) [25]. As shown in Fig. 1e, M15 is more stable to thermal denaturation. It is notable that F15 was the least stable triplex at pH 6.1, which may be due to the compromised basicity of the cytosine-N3 caused by the 2'-fluorination [26]. The respective T_m values are in the order, M15 (71.3°C) > R15 (66.0°C) > F15 (58.4°C) > D15 (54.1°C). These results suggest that the conformation of the 3'-*endo* sugar of the second pyrimidine strand favors a triple-stranded structure, and the increased hydrophobicity of the triplex, caused by the methylation of the 2'-OH, effectively enhances the Hoogsteen bond stability. Examination using a model showed that the phosphate groups of the second pyrimidine strand are in close proximity to the phosphates of the parallel purine strand. The 2'-*O*-methyl groups occupy a narrow groove between their phosphate-sugar backbones. On the other hand, the stability of the triplex R15 was slightly larger than that of M15 at pH 6.1, in contrast to the result at pH 5.0. These results would be mainly explained from a difference in basicity of the cytosine-N3 between R15 and M15.

The formation and stability of the triplexes containing 2'-substituted pyrimidine strands were also confirmed by the gel retardation assay [27]. As shown in Fig. 2, a 15% native polyacrylamide gel, run at pH 5.4, exhibited a band corresponding to the duplex (control, lane 2) and four retarded bands corresponding to the triplexes formed by adding d15, f15, r15, and m15, re-

Table II

Melting temperatures (T_m) of the 15-mer duplex and triplexes

		T_m (°C)	
Complex	Base pair interaction	pH 6.1	pH 5.0
Duplex	Watson–Crick	51.7	48.5
D15	Hoogsteen	28.3	54.1
	Watson–Crick	51.5	
F15	Hoogsteen	20.7	58.4
	Watson–Crick	51.4	
R15	Hoogsteen	37.7	66.0
	Watson–Crick	50.8	
M15	Hoogsteen	36.8	71.3
	Watson–Crick	51.8	

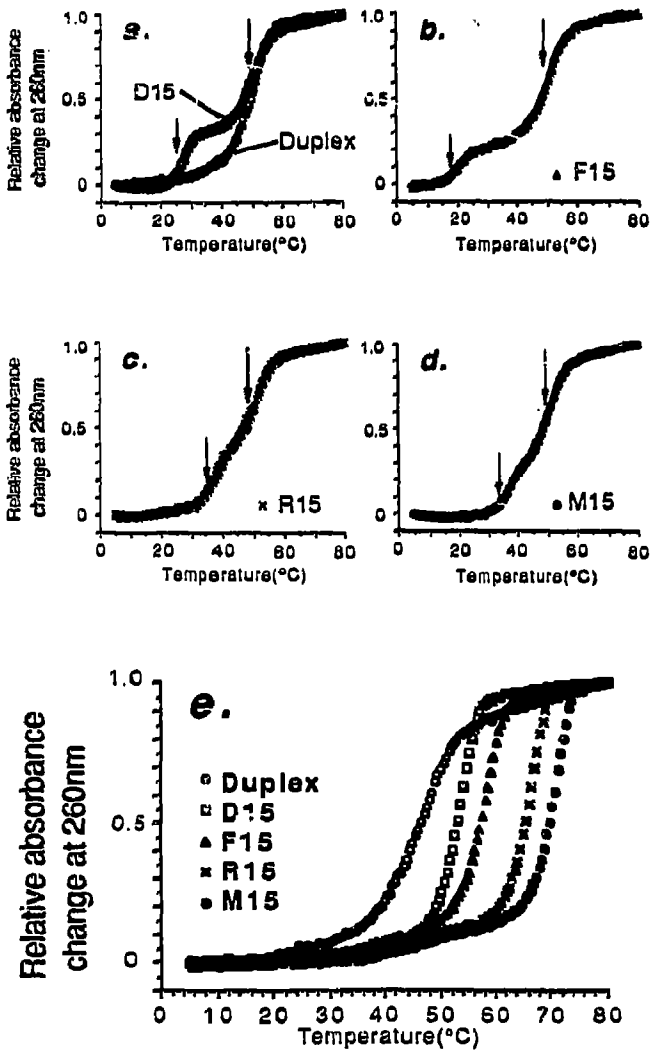

Fig. 1. Relative absorbance change, $[A_{260}(T°C)-A_{260}(5°C)]/[A^{260}80°C)-A_{260}(5°C)]$, with increasing temperature of the 15-mer duplex and the triplexes at pH 6.1 (a–d) and pH 5.0 (e).

spectively, to the duplex containing a ^{32}P-labeled purine strand (lanes 3–6). A band with similar mobility as that in lane 6 was obtained when labeled m15 was used (lane 7). Interestingly, R15 and M15 (lanes 5–7) migrated more slowly than D15 and F15 (lanes 3 and 4). We presumed that this delay reflected the presence of the relatively bulky 2′-substituent in r15 and m15. The instability of the triplex F15, found in the above T_m measurement at pH 6.1 (Table II), was supported by the existence of the strong band corresponding to the remaining duplex in lane 4.

On the other hand, the 2′-*O*-methyl 15-mer strand had greater T_m values than the deoxy 15-mer strand in the melting profiles of triplexes with other AT/GC contents (more AT-rich sequences), as expected (see Table IIIb). In addition, to assess the effect of a mismatch on the stability of a triplex with 2′-*O*-methyl RNA, we examined the thermal stability of a triple-stranded

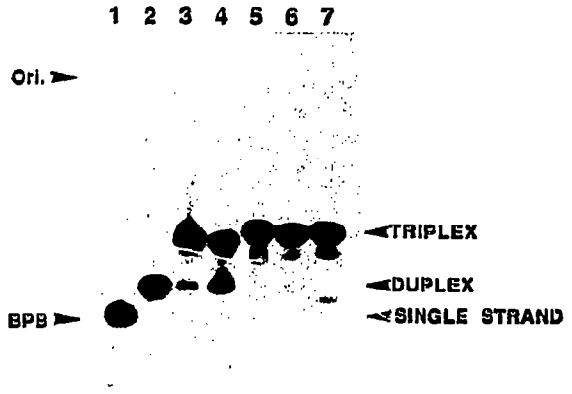

Fig. 2. 15% Non-denaturing polyacrylamide gel electrophoresis.(pH 5.4). Lane 1, dPu*; 2, duplex(dPu*); 3, D15(dPu*); 4, F15(dPu*); 5, R15(dPu*); 6, M15(dPu*); 7, M15(m15*). ^{32}P-Labeled strands are indicated by an asterisk in parentheses.

structure containing one mismatched triplet in the center position of a target 34-mer duplex (Table IIIa) [28]. The 15-mer deoxy strand and the 2′-*O*-methyl strand were separately complexed with 34Pu·34Py to form matched (X=G, Y=C) and mismatched (X=C, Y=G) triplexes. The UV-absorbance-temperature profiles were determined at pH 5.0 in the same manner as in Fig. 1. The T_m values of the Watson–Crick base pairs were almost identical (66.3 ±0.4°C), but the Hoogsteen base pairs melted at different temperatures (Table IIIb). The

Table III

Effect of a mismatched triplet on triplex stability at pH 5.0

(a)

Complex	Sequences
D34	5′TTTCTTTCTTTTCTT 3′ 5′TGAGTGAGTAAAGAAAXAAAAGAATG-AGTGCCAA3′ (34 Pu) 3′ACTCACTCATTTCTTTYTTTTCTTACTC-ACGGTT5′ (34 Py)
M34	5′UmUmUmCmUmUmUmCmUmUmUm-UmCmUmUm3′ 5′TGAGTGAGTAAAGAAAXAAAAGAATG-AGTGCCAA3′ (34 Pu) 3′ACTCACTCATTTCTTTYTTTTCTTACTC-ACGGTT5′ (34 Py)

(b)

	D34		M34	
XY	H.G.	W.-C.	H.G.	W.-C.
GC (matched)	42.4	66.0	50.3	65.9
CG (mismatched)	20.5	66.5	20.3	66.7

(a) Sequences of the oligonucleotides. (b) T_m values (°C) of D34 and M34. H.G. denotes Hoogsteen base pairs and W.-C. denotes Watson–Crick base pairs.

difference (30°C) in the T_m values between the matched and the mismatched M34 triplexes was larger than that (21.9°C) in the D34 series. This important result, although preliminary, shows that 2′-*O*-methyl RNA may be advantageous to discriminate between matched and mismatched triplexes.

In conclusion, the triplexes formed with oligo(2′-*O*-methylribonucleotides) are more thermally stable than those formed by DNA oligomers. 2′-*O*-methyl RNAs are easier to handle, and are relatively nuclease resistant, as compared to RNAs [29]. Therefore, they can be used as anti-gene probes or other biochemical tools. In addition, short triplexes, stabilized by a 2′-*O*-methyl RNA oligomer, may be used to analyze triplex structures by NMR and X-ray crystallographic techniques. Work is underway to convert 2′-*O*-methyl RNA into a gene-targeted artificial nuclease.

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