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Optimization of alternate-strand triple helix formation at the 5'-TpA-3' and 5'-ApT-3' junctions

Priscille Brodin, Jian-Sheng Sun[1], Jean-François Mouscadet* and Christian Auclair

Laboratoire de Physicochimie et de Pharmacologie des Macromolécules Biologiques, CNRS UMR 8532, Institut Gustave Roussy, 39 rue Camille Desmoulins, 94805 Villejuif cedex, France and [1]Laboratoire de Biophysique, UMR 8646 CNRS-Muséum National d'Histoire Naturelle, INSERM U201, 43 rue Cuvier, 75231 Paris cedex 05, France

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ABSTRACT

Alternate-strand triple helix formation was optimized at the two junction steps, the 5'-TpA-3' and 5'-ApT-3' junctions. Footprint experiments, gel retardation assays and thermal denaturation measures on a sequence appropriately designed with two adjacent alternate-strand polypurine tracts points out that the addition of an adenine residue and the removal of one nucleotide should facilitate the crossing strands at the 5'-TpA-3' junction and at the 5'-ApT-3' junction, respectively. These results provide a 'switch code' for the construction of alternate-strand triple helix forming oligonucleotides which open new possibilities for extending the range of applications of antigene strategy.

INTRODUCTION

Although the formation of triple helices with polynucleotides was described as early as 1957 (1), the renewed interest in triple helix formation by oligonucleotides has been aroused since 1987 (2,3) with the realization that synthetic oligonucleotides can be used as antigene agents forming intermolecular DNA triplexes at specific DNA sequences (4–7). In this so-called antigene strategy, oligonucleotides bind to the major groove of the double-stranded homopurine–homopyrimidine sequences in a sequence-specific manner. Three types of triplexes formed with oligonucleotides containing natural bases have been extensively characterized. First, a (C,T)-motif binds the duplex in a parallel orientation but generally requires conditions of low pH (8,9). Second, a (G,A)-motif recognizes the double-stranded target in an antiparallel orientation (10–12). Third, a (G,T)-containing oligonucleotide can also form a (G,T)-motif triple helix in some cases (13). The orientation of the (G,T)-containing third strand is antiparallel or parallel with respect to the target purine strand, depending on the sequence of target double-helical DNA. The orientation of the (G,T)-containing third strand is expected to shift from parallel to antiparallel orientation, with respect to the target polypurine strand, when the number of 5'-ApG-3' and 5'-GpA-3' steps increases in the DNA target sequence and/or when the length of thymine and guanine tracts is changed (14). Beside pH dependency of C.GxC+ base triplets involved in (C,T)-motif triplexes and the isomorphism of base triplets (15), the main restriction of triple helix formation remains that the DNA target sequence must possess all purines on the same strand. However, recent studies have revealed possibilities for extending the range of DNA targets for triple helix formation (for review see 16). Among them, if the targeted sequence is composed of two adjacent and alternating oligopurine–oligopyrimidine tracts, recognition can be achieved by a single third strand, portions of which are targeted against single oligopurine tracts. Such an oligonucleotide zigzags along the major groove, switching from one oligopurine strand to another at the 5'-purine–pyrimidine-3', or 5'-pyrimidine–purine-3' step (hereafter designated as 5'-RpY-3' or 5'-YpR-3' junctions, respectively).

Two distinct approaches have been described to form such a so-called 'alternate strand triple helix' or 'switched triple helix'. On one hand, the two mini triple helices are formed by motifs involving the same hydrogen-bonding interactions (Hoogsteen or reverse Hoogsteen configuration). The use of a suitable linker is therefore required to join the 3'-3' or 5'-5' ends of the mini third strand oligonucleotides through phosphodiester backbone or to bridge between the terminal bases belonging to the adjacent third strands (17–21). On the other hand, the second approach consists of combining alternatively different triple helix motifs involving Hoogsteen and reverse Hoogsteen configurations. In this case, the 5' (or 3') end of one mini third strand oligonucleotide encounters the 3' (or 5') end of its neighbour third strand oligonucleotide at the junction. Thus, artificial linkers are no longer required at the junction of alternating oligopurine–oligopyrimidine tracts, provided that the conformational constraints are properly removed. Consequently, a natural oligonucleotide can bind on such an extended target sequence by formation of a switched triple helix. Previous work using an empirical approach has shown that triple helices can be formed with short third strand oligonucleotides simply linked together by phosphodiester bonds (22–28).

The present work is aimed at optimizing the alternate strand triple helix formation at two sequences which are made of two alternating oligopurine tracts including either a 5'-TpA-3' or a 5'-ApT-3' junction. Based on a preliminary molecular modelling study which highlighted the main structural features at the junctions, we designed appropriate duplexes and third strand oligonucleotides. DNase I footprinting experiments, gel retardation assays and T_m measurements were carried out to

*To whom correspondence should be addressed. Tel: +33 1 42 11 40 07; Fax: +33 1 42 11 52 76; Email: jfm@igr.fr

delineate the code of alternate strand triple helix formation at the 5'-TpA-3' and 5'-ApT-3' junctions.

MATERIALS AND METHODS

Oligonucleotides and plasmids

Oligonucleotides were synthesized on a 391A Applied Biosystems DNA synthesizer and purified by electrophoresis on either 12 or 15% denaturing polyacrylamide/urea gels. Plasmids pBTpA and pBApT containing two adjacent polypurine sequences separated respectively by a 5'-TpA-3' or a 3'-ApT-5' junction were constructed by insertion of complementary oligonucleotides 5'-AATTCTGCAGCCCTCCTTCTCCTTA-AAAGAAAAGGGGGCAGCTCGACG-3' and 5'-GATCCG-TCGAGCTGCCCCCTTTTCTTTTAAGGAGAAGGAGGG-CTGCAG-3' or 5'-AATTCTGCTCGGGAGGAAGAGGAA-TTTTCTTTTCCCCCGAGCTCGACG-3' and 5'-GATCCGT-CGAGCTCGGGGGAAAAGAAAATTCCTCTTCCTCCCG-AGCAG-3' into the *Eco*RI and *Bam*HI sites of pBluescript SK+ vector using standard protocols (29).

Regions of interest in pBTpA and pBApT plasmids were verified by enzymatic sequencing using the Sequenase Version 2.0 protocol (USB, Amersham Life Science) with the 5'-^{32}P-labelled primer (–40) 5'-GTTTTCCCAGTCACGAC-3'.

DNase I footprinting

Footprinting experiments were carried out using DNase I as the cleaving agent. DNA duplexes, referred to hereon as TpA and ApT, were prepared as follows: pBTpA and pBApT were digested with the *Acc*I restriction enzyme, then 3'-labelled with Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP (3000 Ci/mmol), and finally digested with *Sac*I and *Sac*II restriction enzymes, respectively. Labelled fragments were purified on a 2% agarose gel. The 114 and 105 bp labelled DNA duplexes TpA and ApT were incubated overnight at room temperature with increasing concentrations of the third strand in a 45 µl solution containing 10 mM Tris–HCl pH 7.2, 10 mM $MgCl_2$, 0.5 mM spermine and 0.25 µg calf thymus DNA. DNase I (0.05 µg/ml final, Sigma) was added and digestion was allowed to proceed for 6 min at 4°C. The reaction was stopped by adding 10 µl stop solution (10 µg tRNA and 3 M sodium acetate) and 180 µl ethanol. Cleavage products were separated on an 8% polyacrylamide/7 M urea gel and the gel was analyzed on a Molecular Dynamics STORM 840 PhosphorImager.

Gel retardation assays

The 40 bp DNA templates were prepared by *Eco*RI and *Bam*HI digestion of the plasmids pBTpA and pBApT and subsequent 3'-labelling by Klenow polymerase in the presence of 50 µCi [α-^{32}P]dATP. Labelled 40 bp DNA targets were incubated with unlabelled oligonucleotides in a 12 µl solution containing 10 mM Tris–HCl pH 7.2, 10 mM $MgCl_2$, 0.5 mM spermine, 10 µg tRNA and 10% sucrose. After overnight incubation at room temperature, samples were loaded on a 12% non-denaturing polyacrylamide gel. The gel was run for 8 h at 4°C. Analysis and quantification was performed on a Molecular Dynamics STORM 840 PhosphorImager. The uncertainity of quantification was estimated to be ~10% by repeated experiments.

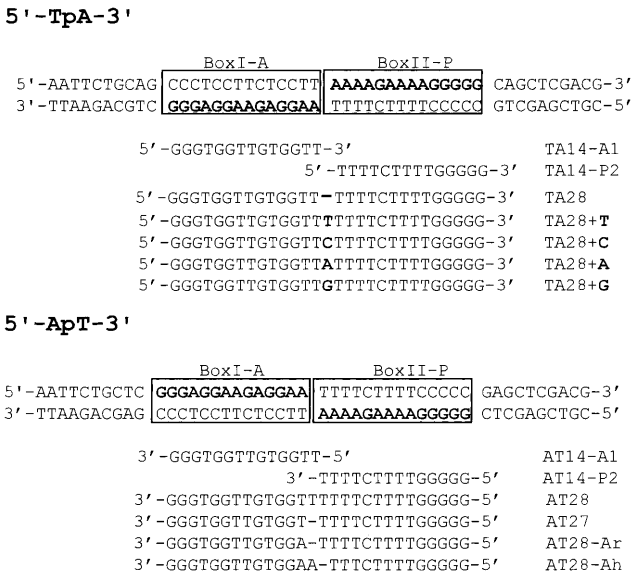

Figure 1. Sequences of oligonucleotides used for optimizing alternate strand triple helix formation at the 5'-TpA-3' and 5'-ApT-3' junction step.

UV absorption spectroscopy

Thermal denaturation profiles were recorded with a Uvikon 933 spectrophotometer with 1 µM of each strand of the target DNA and 1.5 µM of the third strand in 10 mM cacodylate buffer at pH 7.0 with 10 mM $MgCl_2$. The temperature of the bath was increased or decreased at a rate of 0.1°C/min from 4 to 94°C. Melting temperatures ($T_m^{3\rightarrow2}$) were evaluated as the temperature at which half-association (or dissociation) of the third strand from the double-helical target occurred and were estimated directly from first derivative curves.

RESULTS

A set of (G,T) oligonucleotides was designed to form alternate strand triple helices with either a 5'-CCCTCCTTCTCCT-**TA**AAAGAAAAGGGGG-3' or a 5'-GGGAGGAAGAGGA-**AT**TTTCTTTTCCCCC-3' target sequence containing either a 5'-TpA-3' or a 5'-ApT-3' junction step. Sequences of the oligonucleotides used in this study are given in Figure 1. Binding of these oligonucleotides requires the formation of two triplex motifs: the first triple-helical domain is a (G,T)-motif triplex in reverse Hoogsteen configuration with the third strand bound in an antiparallel orientation with respect to the left-half target purine GGGAGGAAGAGGAA (Box I-A) and the second one involves a (G,T)-motif triplex in Hoogsteen configuration with the third strand bound in a parallel orientation with respect to the right-half target purine AAAAGAAAAGGGGG (Box II-P).

5'-TpA-3' junction step

The switch (T,G) oligonucleotides are composed of the TA14-A1 and the TA14-P2 sequences linked together with or without additional nucleotide. DNase I footprinting experiments were first carried out to determine whether triplex formation

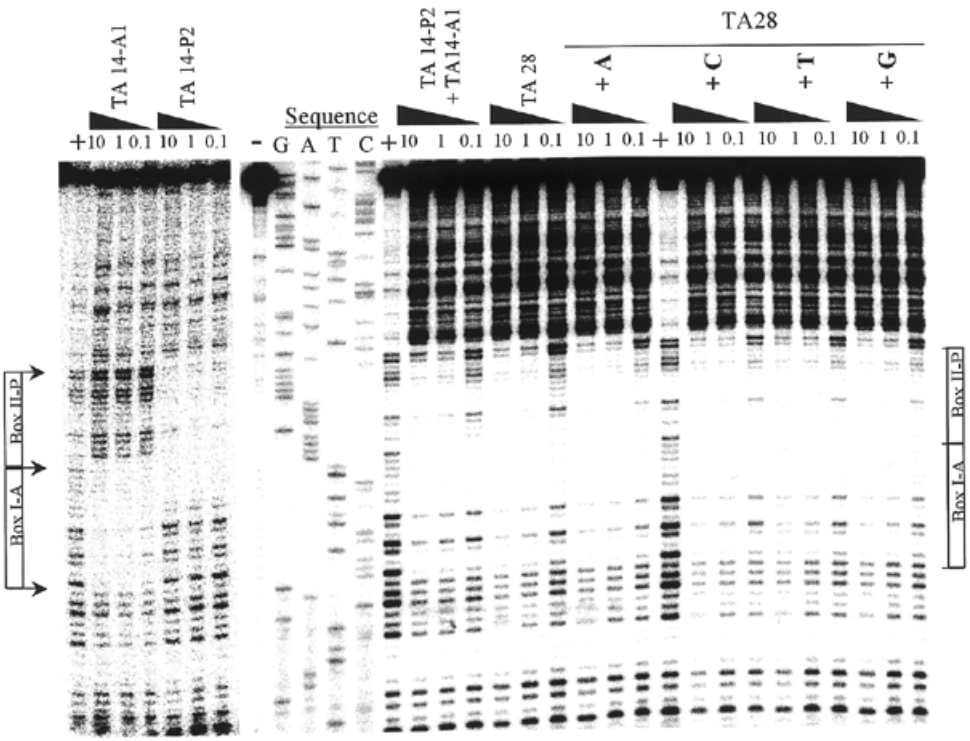

Figure 2. DNase I footprinting experiments of switch oligonucleotides bound to the alternating oligopurine–oligopyrimidine double-helical sequences containing a 5'-TpA-3' step.

occurred (Fig. 2). The parallel (Box II-P) and antiparallel domain (Box I-A) were fully protected in the presence of TA14-P2 and TA14-A1, respectively. In the presence of any of the five third strand oligonucleotides, simultaneous protection of both Box I-P and Box II-A was observed. It must be noted that the extent of simultaneous protection of both domains increases in a concentration-dependent manner. These observations provide evidence of an alternate strand triple helix formation on both strands.

Gel retardation experiments were performed to reinforce this conclusion. As can be seen in Figure 3, only one major shifted product was observed upon binding of the third strand oligonucleotide. To compare the stability of the different triplexes, quantitative gel retardation assays were performed. Triplex formation could be observed with the 14 nt third strand AT14-P2 which binds to the polypurine tract in the parallel orientation, whereas no shifted product was observed up to 20 μM with the 14 bp AT14-A1 which binds the purine target sequence in the antiparallel orientation (Table 1). The 28 nt switch oligonucleotides show a much higher affinity for the duplex than the 14 nt TA14-A1. Moreover, as can be readily observed from Figure 3, the four oligonucleotides that contain an additional base display higher affinity for alternating 5'-$(Py)_{13}TpA(Pu)_{13}$-3' than switch oligonucleotide TA28. In our experimental conditions, there was a great excess of triple helix forming oligonucleotide (TFO) with respect to the labelled target. Under these conditions, the estimation of apparent K_d is the value of TFO concentration when half of the target is shifted. The estimated K_d values are summarized in

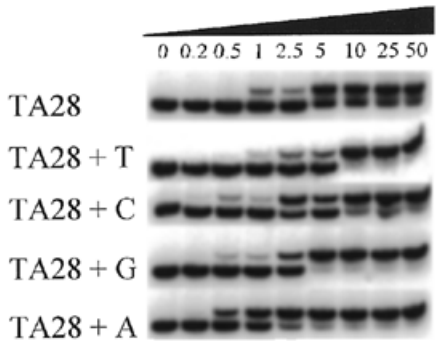

Figure 3. Gel retardation assays for the binding of switch oligonucleotides to the alternating oligopurine–oligopyrimidine double-helical sequences containing a 5'-TpA-3' junction step.

Table 1. The addition of a purine provides better binding than that of a pyrimidine, and it appears that A is better than G.

UV absorption experiments were also performed. Melting curves for each of the four TFOs show two transition steps. One transition occurring at 78°C is common to all samples and corresponds to the dissociation of the duplex target into single strands ($T_m^{2\rightarrow1}$). The other transition corresponds to the dissociation of the triplex into a duplex and a free third strand ($T_m^{3\rightarrow2}$). T_m values for triplex dissociation depend upon the third strand sequence (Fig. 4). $T_m^{3\rightarrow2}$ of triplexes with a third strand containing an additional purine (TA28+G and TA28+A) were higher than with the one containing an additional pyrimidine (TA28+T and TA28+C). As control, we checked that the third

Table 1. Dissociation binding constants (K_d) determined by gel retardation assays and melting temperatures $T_m^{3\rightarrow2}$ of the switch oligonucleotides targeted to the alternating oligopurine–oligopyrimidine sequences containing either a 5'-TpA-3' (top part) or a 5'-ApT-3' (bottom part) junction step

Junction	Oligonucleotide	K_d (μM)	$T_m^{3\rightarrow2}$ (°C)
5'-TpA-3'	TA28	3.8	47
	TA28+T	3.0	53
	TA28+C	2.5	54
	TA28+G	1.3	59
	TA28+A	0.9	60
5'-ApT-3'	AT28	4.5	40
	AT27	2.2	45
	AT28-Ar	1.2	50
	AT28-Ah	1.1	52

The uncertainity of the K_d value is estimated to ~10%.

strand oligonucleotides alone do not show any transition step. Furthermore, third strand oligonucleotides incubated with the 40 bp duplex in the opposite orientation (5'-ApT-3') do not give rise to a $T_m^{3\rightarrow2}$.

5'-ApT-3' junction step

Figure 1 shows the third strand (T,G) oligonucleotides that were made of both AT14-A1 and the AT14-P2 sequences in which one of the central nucleotides may be removed. As shown in Figure 4, DNase I footprinting experiments show that Box II-P and Box I-A are effectively recognized by oligonucleotides AT14-P2 and AT14-A1, respectively. A moderate protection of both Box II-P and Box I-A was also observed when both oligonucleotides were added simultaneously. The 28 nt oligonucleotide (AT28) was able to give footprint on both boxes, in particular on Box II-P. The 27 nt oligonucleotides with one nucleotide removed (AT27 and AT28-Ah) provided a higher extent of protection than the 28 nt oligonucleotide. These data provide evidence of alternate strand triplex formation at the 5'-ApT-3' junction.

Gel retardation assays allowed us to compare quantitatively the stability of different triplexes. Triplex formation could be observed with the 14 nt third strand AT14-A1 which binds to the polypurine tract in the antiparallel orientation, whereas no shifted product was observed in our experimental conditions with the 14 bp AT14-P2 which binds the purine target sequence in the parallel orientation (Fig. 5). Moreover, with all 28 or 27 bp third strands, a clear band shift corresponding to alternate triple helix formation was observed. As previously reported, formation of such triplexes requires the presence of a high concentration of magnesium. In this study, a minimum of 10 mM $MgCl_2$ was necessary to observe a clear band corresponding to the triplex.

The removal of one thymine from the middle of the oligonucleotide AT27 led to a 2-fold increase of TFO affinity as compared with the parent 28 nt AT28 (Table 2). The affinity of TFO was further increased ~2-fold when a T at the 5'-end of the reverse Hoogsteen was substituted by an A (AT28-Ar). The addition of an A at the 5'-end of the reverse Hoogsteen domain and the removal of a T at the 3'-end of the Hoogsteen domain (AT28-Ah) gave rise to a roughly equal binding constant.

Table 2. Proposed 'switch' code for alternate strand triple helix formation at all six junction steps junctions

Junction steps	Target sequences	TFO sequences	References
5'-YpR-3':			
5'-TpA-3'	5'-ttAA-3'	5'-tt*A*TT-3' or	this work
	3'-aaTT-5'	5'-aa*A*TT-3'	
5'-CpG-3'	5'-ccGG-3'	5'-gg*C*GG-3'	31
	3'-ggCC-5'		
5'-CpA-3'	5'-ccAA-3'	5'-gg*CC*TT-3'	32
(5'-TpG-3')	3'-ggTT-5'		
5'-RpY-3':			
5'-ApT-3'	5'-AAtt-3'	5'-TTa-3' or	this work
	3'-TTaa-5'	5'-Taa-3' or	
		5'-TTt-3'	
5'-GpC-3'	5'-GGcc-3'	5'-GGgg-3'	31
	3'-CCgg-5'		
5'-GpT-3'	5'-GGtt-3'	5'-GGa-3' or	32
(5'-ApC-3')	3'-CCaa-5'	5'-GGt-3'	

The upper and lower case letters indicate that the sequences are involved in Hoogsteen or reverse Hoogsteen domains, respectively. The italic letters show the nucleotides used as linkers to bridge the gap between Hoogsteen and reverse Hoogsteen-like parts of the third strand. Divalent cations are required to form a stable triple helix at neutral pH.

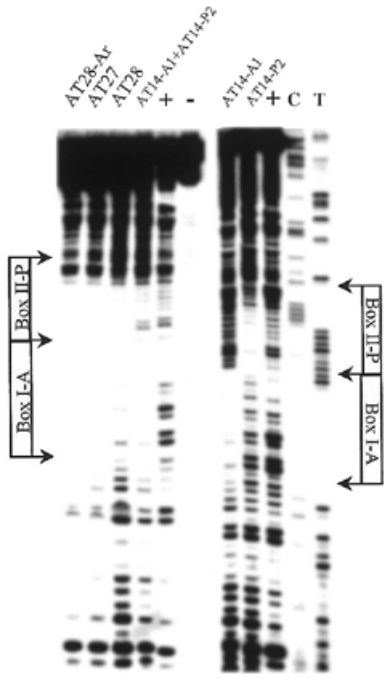

Figure 4. DNase I footprinting experiments of switch oligonucleotides bound to the alternating oligopurine–oligopyrimidine double-helical sequences containing a 5'-ApT-3' step.

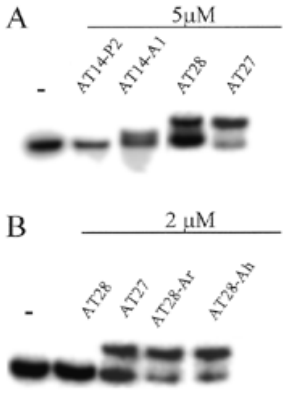

Figure 5. Gel retardation assays for the binding of switch oligonucleotides to the alternating oligopurine–oligopyrimidine double-helical sequences containing a 5'-ApT-3' junction step at 5 μM (**A**) and at 2 μM (**B**).

In parallel, UV absorption experiments were also performed. As for the 5'-TpA-3' junction, derivative curves showed two transition steps. For all third strands, $T_m^{2\rightarrow1}$ occurs at 78°C. $T_m^{3\rightarrow2}$ values, which depend on the third strand, are summarized in Table 1. The temperature of dissociation $T_m^{3\rightarrow2}$ of the triplex with a third strand missing a thymine (AT27) was higher than the one corresponding to the exact sequence. The triplex containing an A instead of a T on the 3' side of the junction (AT28-Ah) as well as on the 5' side of the junction (AT28-Ar) gave rise to a higher $T_m^{3\rightarrow2}$.

DISCUSSION

Alternate strand triple helix formation has been previously reported in the literature (30). It was shown that crossing over of the third strand at the 5'-RpY-3' junction can be achieved by directly attaching Hoogsteen and reverse Hoogsteen parts of the third strand oligonucleotides without any additional linker, whereas an appropriate linker should be required at the 5'-YpR-3' junction (31,32). A preliminary molecular modelling study performed on 5'-ApT-3' and 5'-TpA-3' junctions, led to similar conclusions (data not shown). Although our results are consistent with the general conclusions for alternate strand triple helix formation, the optimization of the so-called 'switch' oligonucleotides (TFOs) carried out in the present study reveals some sequence-specific effects which depend on the sequence context of both the junction and the combination of involved triple helix motifs. This work shows by experimental methods such as DNase I footprinting, gel retardation assay and T_m measurements that (i) an additional adenine in the third strand oligonucleotide at the 5'-TpA-3' junction, and (ii) the removal of one nucleotide at the 5'-ApT-3' junction, provide stronger binding than the oligonucleotide made by directly attaching two adjacent Hoogsteen and reverse Hoogsteen binding domains. In fact, the optimization of the TFO sequences at the junctions leads to an increase of ~4-fold in binding constant (K_d) and ~12–13°C in T_m (Table 1) as compared with the simple head-to tail linked TFO. Moreover, K_d values are much higher for 28 nt switch oligonucleotides than the corresponding 14 nt oligonucleotides, which points out another advantage of long switch oligonucleotide over shorter ones. Molecular modelling indicates that the stabilization at the 5'-ApT-3' junction is ascribed to a better intra-third strand base stacking along with a significantly reduced deformation energy of the target duplex.

During the course of the current study, two similar works have been reported on the optimization of alternate strand triple helix formation at other junctions steps (i.e. 5'-CpG-3'; 5'-GpC-3'; 5'-TpG-3'/5'-ApC-3' and 5'-GpT-3'/5'-ApC-3') (31,32). Taking into account this published data and the data shown in this work, we are tentatively establishing a 'switch' code (Table 2) aimed at providing a rational basis to design appropriate TFO sequence which can form the most stable alternate strand triple helix at any six junction steps. This should help to extend the repertory of double-stranded DNA sequences which can be recognized by TFOs and to provide a new basis for the development of the 'antigene' strategy in order to specifically control gene expression.

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