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Synthesis and Properties of Triple Helix-Forming Oligodeoxyribonucleotides Containing 7-Chloro-7-deaza-2′-deoxyguanosine

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Abstract—Multiple incorporations of 7-chloro-7-deaza-2′-deoxyguanosine in place of 2′-deoxyguanosine have been performed into a triple helix-forming oligodeoxyribonucleotide involving a run of six contiguous guanines designed to bind in a parallel orientation relative to the purine strand of the DNA target. The ability of these modified oligodeoxyribonucleotides to form triple helices in a buffer containing monovalent cations was studied by UV–melting curves analysis, gel shift assay and restriction enzyme protection assay. In the presence of Na^+, the incorporation of two, three or five modified nucleosides in the third strand has improved the efficacy of formation of the triplex as compared to that formed with the unmodified oligonucleotide. The stabilities of the three modified triplexes were similar. The coupling of 6-chloro-2-methoxy-9-(ω-hexylamino)-acridine to the 5′-end of the oligonucleotides containing modified nucleosides led to an increase in triplex stability similar to that observed when the acridine was added to the 5′-end of the unmodified oligonucleotide. In the presence of K^+, only the oligodeoxyribonucleotides containing modified G retained the ability to form triple helices with the same efficiency. The incorporation of the modified nucleoside has two effects: (i) it decreases TFO self-association, and (ii) it slightly increases triplex stability. The enhanced ability of the modified oligonucleotides containing 7-chloro-7-deaza-2′-deoxyguanosine over the parent oligomer to form triple helices was confirmed by inhibition of restriction enzyme cleavage using a circular plasmid containing the target sequence.

Introduction

The first report of nucleic acids triple helix formation in 1957 by Felsenfeld and Rich,[1] followed 30 years later by the discovery that synthetic oligonucleotides could recognize short oligopyrimidine•oligopurine runs in double-stranded DNA,[2,3] via hydrogen bond formation with purine bases in the major groove, have promoted numerous studies on the use of triple helix-forming oligonucleotides (TFOs) because of their possible applications in biotechnology, diagnostics and therapeutics (for reviews, see ref 4). Short triple-stranded complexes can inhibit transcription initiation by interfering with the binding of transactivating proteins,[5] and a triplex formed downstream from a promoter can block transcription elongation.[6,7] In addition, reactive TFOs have been shown to induce targeted mutations,[8,9] and conjugates of oligonucleotides with psoralen and alkylating agents have been used to demonstrate that the DNA target sequence is accessible in the cell nuclei.[10,11] A TFO covalently linked to camptothecin, an inhibitor of topoisomerase I, induces in vitro sequence-specific cleavage of one strand of the double-stranded DNA target by recruiting the topoisomerase I enzyme.[12,13]

Three motifs have been described for the recognition of oligopurine runs by TFOs. A pyrimidine third strand binds parallel to the purine strand of the double-stranded target to form T•AxT and C•GxC$^+$ isomorphous triplets via Hoosgsteen hydrogen bonding (where C$^+$ indicates protonated cytosine and the symbols • and x stand for Watson–Crick and Hoogsteen hydrogen bonds, respectively). The binding stability of the C/T-containing pyrimidine third strand is pH sensitive because its cytosine bases can only form Hoogsteen bonds when protonated rendering the targeting of C•G

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stretches difficult due to the charge–charge repulsion between contiguous protonated cytosines. 5-Methylcytosine is frequently substituted for cytosine in order to increase triplex stability with the (T,C)-containing third strand. A purine third strand binds in an anti-parallel orientation forming C•GxG ant T•AxA base triplets via reverse Hoogsteen hydrogen bonding. G- and T-containing oligonucleotides can also form triplexes whose third strand orientation is sequence dependent.[14] The stability of these two families of G-containing oligonucleotides is hampered by their tendency to self-associate in the presence of monovalent K^+. Some G-containing TFOs can form aggregates stabilized by guanine quartets[15,16] potentially restricting the effectiveness of purine-rich TFOs as antigene reagents. To overcome this problem, several analogues of guanine have been incorporated into TFOs. Among these analogues, 7-deaza-2′-deoxyguanosine[17] whose N(7) H-bond acceptor was replaced by a C–H group, and 9-deaza-2′-deoxyguanosine[18] whose N(7) H-bond acceptor was replaced by an N-H group, were used to disrupt the H-bonding network of the G-tetrad by removing the Hoogsteen hydrogen bonds between the 2-amino and N (7) groups while retaining the hydrogen bond donor and acceptor pattern for the formation of C•GxG triplets. However, the use of these modified nucleosides to overcome the binding inhibition by the monovalent K^+ cation and consequently to increase triplex formation was not as successful as expected. Another approach consists in replacing guanosine by a nucleoside analogue to introduce a steric hindrance. The use of 6-thioguanine (S6-dG)[19] in which the O(6) oxygen was replaced by the less electronegative and larger sulfur atom was expected to decrease the tetrad stability. (S6-dG)-containing triple helices are no longer sensitive to potassium but, however, are less stable than those containing non-modified dG.

We report here multiple incorporations of the previously described 7-chloro-7-deaza-2′-deoxyguanosine;[20] to our knowledge, it has not been incorporated into oligonucleotides until now, in place of 2′-deoxyguanosine. Here we have modified a TFO containing a run of six contiguous G, and designed to bind in the parallel orientation relative to the purine strand of the DNA target (Fig. 1). For triple helix formation, we have

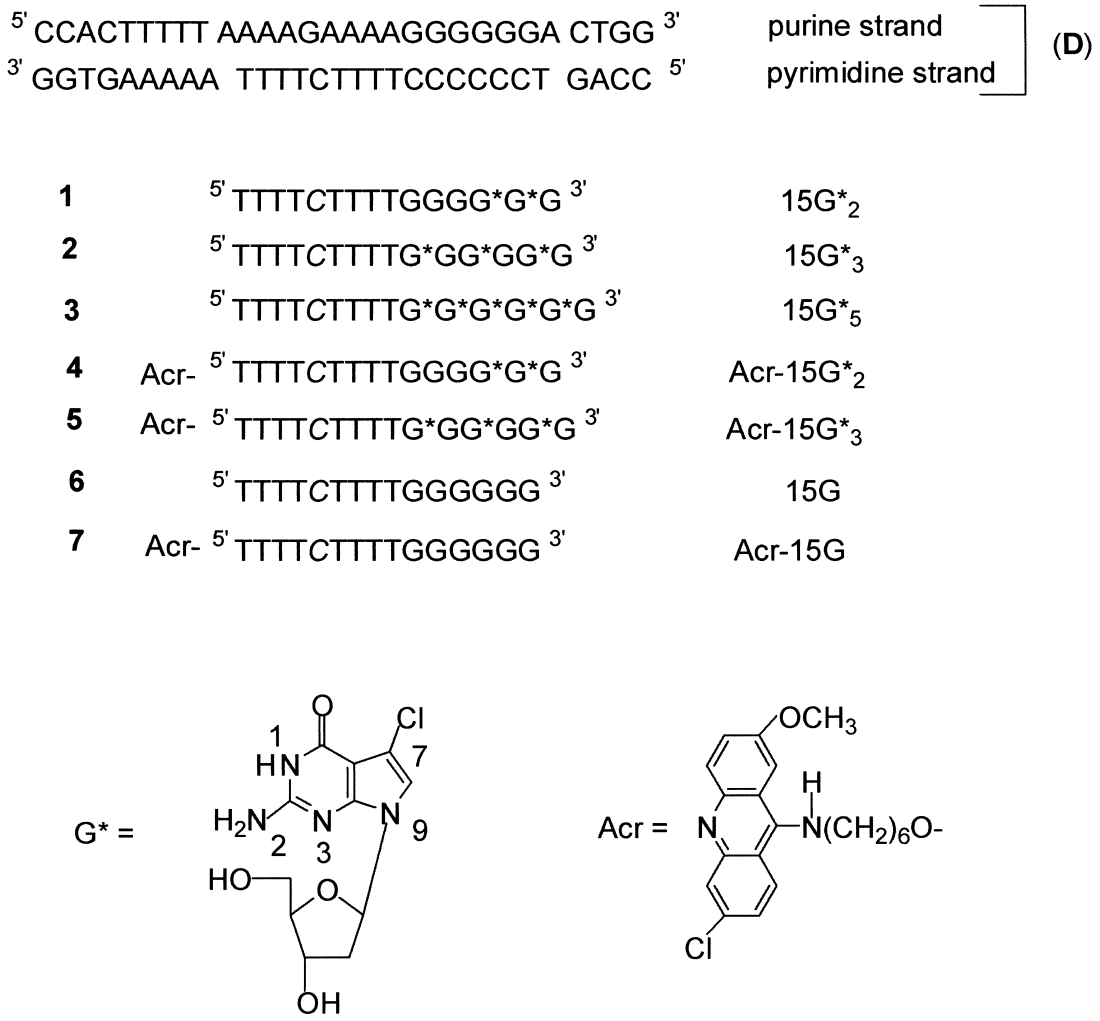

Figure 1. Chemical structures of triple-helical complexes. The sequences of the 29-bp long double-stranded target containing the 16-bp long PPT oligopyrimidine•oligopurine sequence, and of the third strands are depicted. The modified nucleoside used G* is presented to the left. *C* stands for 5-methyl-2′-deoxycytidine. Acr indicates 2-methoxy-6-chloro-9-amino acridine attached to the 5′-phosphate via its 9-amino group and a hexamethylene linker.

chosen as a target a 16 bp oligopyrimidine•oligopurine sequence, present twice on HIV-1 DNA in the *integrase* and in *nef* viral genes. The coupling of an intercalating agent 6-chloro-2-methoxy-9-(ω-hexylamino)-acridine, has been performed to the 5′-end of TFOs containing 7-chloro-7-deaza-2′-deoxyguanosines. The ability of these modified oligonucleotides to form triple helices in a monovalent cation-containing buffer has been studied by gel shift assay, UV–melting curve analysis and by protection to restriction enzymatic cleavage.

Results and Discussion

Experimental design

As a target for triple helix formation, we have chosen a 16 bp oligopyrimidine•oligopurine sequence present on proviral HIV-1 DNA. This run is depicted in Figure 1 as part of a synthetic 29 bp long duplex (D). Triplex-forming oligonucleotides (TFOs) specific to this oligopurine run are aligned below it. Two of these oligonucleotides were conjugated at their 5′-terminus to an intercalating agent, 2-methoxy-6-chloro-9-amino acridine via its 9-amino group and an hexamethylene linker (the acridine-linker derivative is represented by Acr).

Synthesis of modified nucleotide 7-choro-7-deaza-2′-deoxyguanosine and preparation of its phosphoramidite derivative

The 7-chloro-7-deaza-2′-deoxyguanosine was prepared according to a previously published procedure.[20,21] After protection of the exocyclic amino function with isobutyryl and 5′-hydroxyl function with dimethoxytrityl groups, the 7-chloro-7-deaza-2′-deoxyguanosine was transformed into its phosphoramiditive derivative following a published procedure.[22]

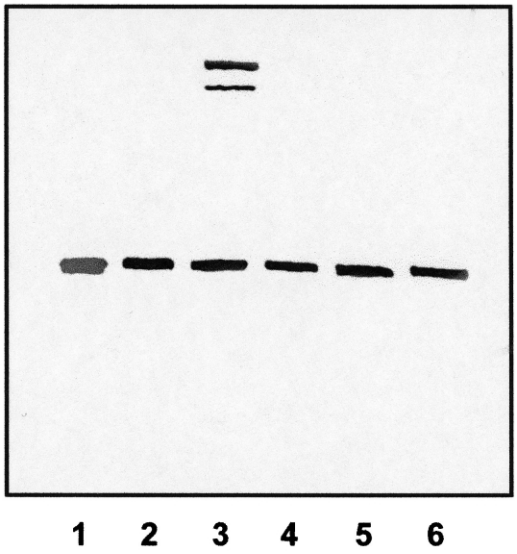

Figure 2. Denaturing polyacrylamide (20%) gel electrophoresis of modified and unmodified oligodeoxyribonucleotides stained with methylene blue: lane 1: (15 G_2^*), lane 2: (15 G_3^*), lane 3: (15 G), lane 4: (15 G_5^* fraction 1 with Rt = 16 min, 51 s), lane 5: (15 G_5^* fraction 2 with Rt = 22 min, 9 s), lane 6: (15 G*$_5$, fraction 1 + fraction 2). (See Experimental for definition of fraction 1 and fraction 2).

Oligonucleotide synthesis

Oligonucleotides **1**–**3** containing multiple incorporations of the modified nucleoside, 7-chloro-7-deaza-2′-deoxyguanosine, were prepared via phosphoramidite chemistry as well as oligonucleotides **4** and **5** containing both the modified nucleoside and an intercalating agent, 6-chloro-2-methoxy-9-(ω-hexylamino)-acridine, linked to their 5′-ends. Oligonucleotides **2**, **4** and **5** were characterized by nuclease degradation analysis which confirmed the presence and the number of the modified nucleoside 7-chloro-7-deaza-2′-deoxyguanosine. Electrospray mass spectroscopy analysis of oligonucleotides **1**, **2**, **3** and **5** confirmed their mass. In the case of the modified oligonucleotide **3** containing five contiguous incorporations of the modified nucleoside 7-chloro-7-deaza-2′-deoxyguanosine, two peaks were obtained during the analyses and purification steps by liquid chromatography. The products corresponding to the two peaks were collected separately and analyzed by electrospray mass spectroscopy. The same mass was obtained for both compounds.

PAGE analysis confirmed that the incorporation of the modified nucleotide prevents the formation of G quartets in the conditions used (Fig. 2). In the case of the modified oligodeoxyribonucleotide **3** (15 G_5^*), the same mobility was observed for the two compounds collected separately during the purification.

Binding analysis: T_m measurements by UV absorption and gel shift assay

DNA thermal dissociation experiments were carried out with the various triplexes containing a modified third strand, in the presence of 10 mM $MgCl_2$ and 50 mM NaCl. The triplex containing the unmodified third strand was used as reference. Oligonucleotides containing multiple incorporations of 7-chloro-7-deaza-2′-deoxyguanosine at different positions inside the **1**–**3** sequences formed triple helices with a 5 °C increase in melting temperature over the triple helix formed with the parent oligonucleotide containing the natural 2′-deoxyguanosine (Table 1). Two, three or five incorporations of the modified nucleoside improved the stability of the triplex to the same extent indicating that the effect was not additive for multiple incorporations. These observations were confirmed by gel shift analysis (Fig. 3). In this study oligonucleotides **1**–**3** (15$G^*_{2,\ 3\ or\ 5}$) bind with quite similar affinities at 25 °C but largely higher than that of the unmodified oligonucleotide **6** (15G). At a 10 μM concentration, 100% triplex was

Table 1. Effect of monovalent cations[a]

Oligo	15$G^*_{2,3\ or\ 5}$	Acr-15$G^*_{2\ or\ 3}$	15G	Acr-15G
(Na^+)	35	50	30	47
(K^+)	34	48	nd[b]	nd

[a]Melting temperature T_m (°C) (±1 °C) was determined in two conditions: a 10-mM sodium cacodylate buffer (pH 6.5) was used containing 10 mM $MgCl_2$ and either 50 mM NaCl (Na^+) or 150 mM KCl (K^+). For other conditions see Materials and Methods
[b]nd, not detected

formed with the G-modified TFO ($15G^*_2$), whereas only 15% triplex was detected for the unmodified 15G in the same conditions (Fig. 3, lanes 5). The specificity of triplex formation with the modified TFOs was studied by gel shift assay using a mutated double-stranded target sequence (5′ AAAAGAAAAaGGaGGA 3′) with two mismatches in the purine run. No triplex could be detected with the G-modified TFOs at 10 μM concentration that allowed to observe complete triplex formation with the wild-type target.

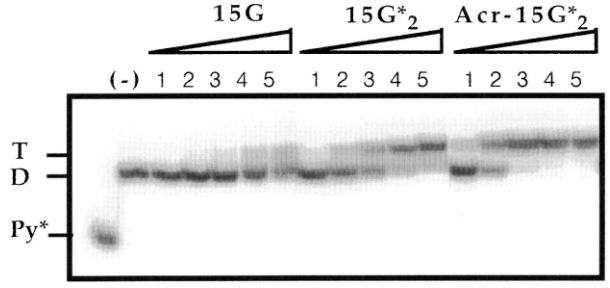

Figure 3. Non-denaturing gel analysis of triplex formation by modified $15G^*_2$ and Acr-$15G^*_2$ triple-helix forming oligonucleotides (TFO) targeted to the polypurine tract of HIV-1, as indicated. A concentration series of each TFO was incubated with labeled 50 nM duplex in 50 mM MES buffer, pH 6, containing 50 mM NaCl, 10 mM $MgCl_2$ and 10% sucrose, at 25 °C. Reaction samples were electrophoresed in 15% non-denaturing polyacrylamide gel (50 mM MES pH6, 10 mM $MgCl_2$) at 25 °C. TFO concentration in aliquots applied to lanes 1–5 were 0.1, 0.5, 1, 5 and 10 μM, respectively. The same results are obtained with the different G-modified oligonucleotides ($15G^*_{3,5}$ and Acr-$15G^*_3$). Py*: 5′- [γ^{32}P] labeled pyrimidine target strand; D, duplex; T, triplex.

The coupling of 6-chloro-2-methoxy-9-(ω-hexylamino)-acridine to the 5′-end of the oligonucleotide containing two or three modified nucleosides **4** or **5** (Acr-$15G^*_{2\text{ or }3}$) led to an increase in T_m of about 15 °C similar to that observed when the acridine was added to the 5′-end of the unmodified oligonucleotide **7** (Acr-15G) (Table 1). Gel shift analysis confirmed that the covalent coupling of the acridine derivative to the 5′-end of the oligonucleotide containing the modified 2′-deoxyguanosine increases its binding affinity (Fig. 3). At 1 μM 100% triplex was formed with the acridine conjugate (Acr-$15G^*_2$) and around 35% with the unconjugate TFO ($15G^*_2$) (Fig. 3, lanes 3).

Triplex formation was then studied by absorption spectroscopy in more physiological conditions, that is in the presence of 150 mM KCl instead of 50 mM NaCl as described above. For the oligonucleotides containing the modified G ($15G^*_{2,3\text{ or }5}$, Acr-$15G^*_{2,3}$), two transitions were still observed reflecting the dissociation of the TFO at low temperature, then the melting of the double-stranded target at a higher temperature (Fig. 4). In contrast, for the unmodified TFO (15G) in the presence of K^+ ions, only the duplex melting profile was detected. It is likely that under these conditions, the 15G oligonucleotide was mainly involved in a multistranded structure and therefore no longer available for triplex formation.

There is a two-sided effect when the modified nucleoside is incorporated into the third strand of the triplex: (i) it slightly increases triplex stability by itself (as suggested by the T_m values in the Na^+-containing buffer); (ii) it decreases the TFO self-association thus increasing the

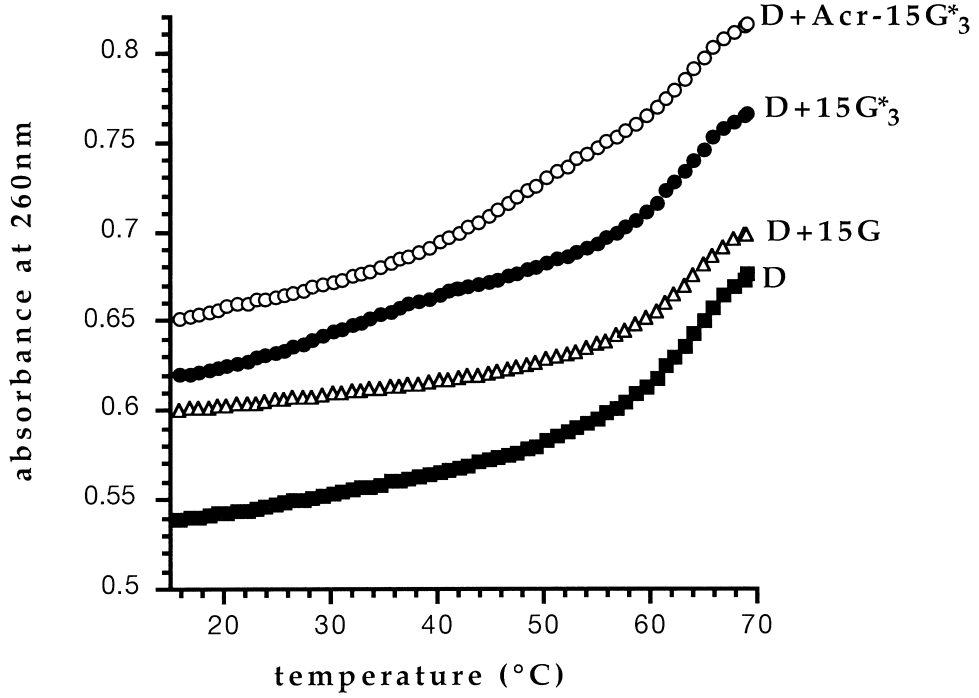

Figure 4. Triplex stability: absorbance measurements. Melting profiles of the triplex formed by the various TFOs and the 29-bp long double-stranded target (D) ((Δ) 15 G, (●) $15G^*_3$, (○) Acr-$15G^*_3$) (see Fig. 1 for sequences). The T_m measurements were conducted in 10 mM sodium cacodylate, pH 6.5, 150 mM KCl and 10 mM $MgCl_2$ as described in Materials and Methods. The different G-modified oligonucleotides provided equivalent results ($15G^*_2$, $15G^*_3$ and $15G^*_5$ behave similarly as do Acr-$15G^*_2$ and Acr-$15G^*_3$).

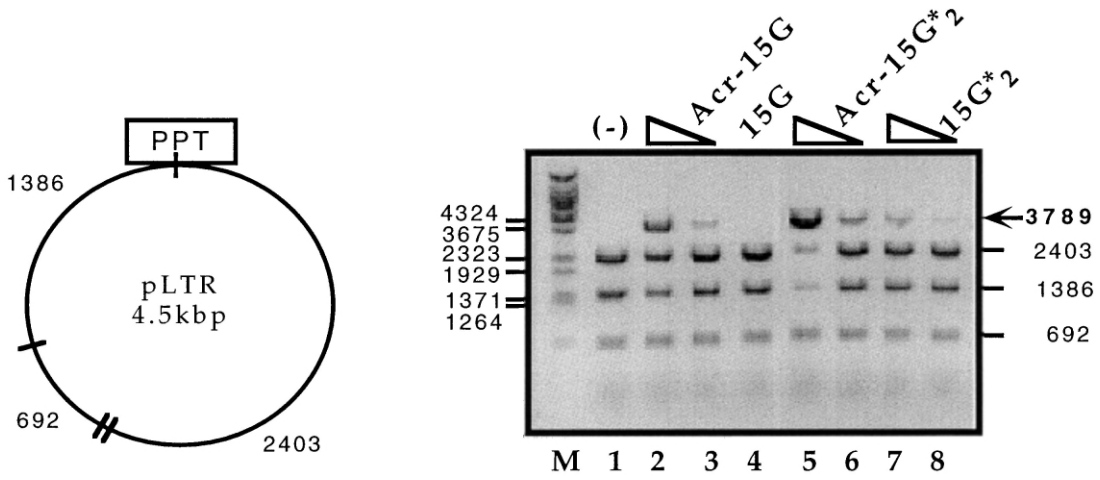

Figure 5. Specific inhibition of Dra I cleavage. Left: Schematic restriction map of pLTR plasmid. The positions of Dra I cleavage sites on the plasmid are indicated together with the lengths of the cleaved fragments. The triple-helix site PPT is boxed. Right: The supercoiled pLTR plasmid (2γ/10λ) was incubated with various TFO as indicated in 50 mM Hepes buffer (pH 7.2) containing 50 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM spermine. Dra I cleavage was performed for 25 min at 37 °C. The different G-modified TFOs ($15G^*_2$, $15G^*_3$ and $15G^*_5$) exhibited Dra I cleavage inhibition to the same extent as did the two acridine conjugates (Acr-$15G^*_2$ and Acr-$15G^*_3$). lane 1: no oligo; lanes 2–3: Acr15G (0.5–0.1 μM); lane 4: 15G (10 μM); lanes 5–6: Acr $15G^*_2$ (0.5–0.1 μM); lanes 7–8: $15G^*_2$ (0.5–0.1 μM); lane M: λDNA digested with Bst E II.

effective concentration of TFO available for triplex formation (as suggested by T_m values in the K^+-containing buffer). For both reasons, G modification is favorable to triplex formation.

Specific inhibition of enzymatic cleavage

The enhanced ability of the modified oligonucleotide containing 7-chloro-7-deaza-2′-deoxyguanosine over the parent oligonucleotide to form triple helices was confirmed by inhibition of restriction enzyme cleavage using a circular plasmid containing the target sequence. We used the pLTR plasmid as a target for triplex-forming oligonucleotides directed against the PPT/HIV-1 sequence.[23] It contains 4 Dra 1 cleavage sites (TTT↓AAA), and the Dra 1 recognition sequence overlaps the 16-mer oligonucleotide binding site by three base pairs at only one of these sites (see Figure 5). In the absence of TFO, the cleavage reaction products have lengths of 1386, 2403, 19 and 692 bp. All these fragments were observed on an agarose gel, except for the short 19 bp fragment. If triplex formation at the PPT site inhibits binding and/or cleavage by Dra 1 at this site, the lengths of the generated fragments should be 2403 + 1386 = 3789, 692 and 19.

The supercoiled pLTR plasmid was incubated in the presence of various modified and unmodified oligonucleotides directed against the PPT sequence, in order to evaluate their relative efficiency in competing with the enzymatic cleavage. When using 0.5 μM of the oligonucleotide containing modified nucleotides and bearing the acridine derivative at the 5′-end 70% inhibition was observed (Fig. 5, lane 5) while the oligonucleotide-acridine with the natural 2′-deoxyguanosine caused only 30% inhibition (Fig. 5, lane 2). The oligonucleotide involving the modified nucleoside but without the intercalating agent caused only 10% inhibition (Fig. 5, lane 7) while no inhibition could be detected in the presence of the unmodified oligonucleotide (Fig. 5, lane 4). As expected the efficiency of the inhibition of restriction enzyme cleavage is correlated to the triplex stability described above.

Conclusion

We have described a multiple incorporations of 7-chloro-7-deaza-2′-deoxyguanosine into a TFO containing a run of six contiguous G. The unmodified TFO is involved in multi-stranded structures that are largely inhibited in the presence of modified G. In addition, the TFO with modified G is able to form a more stable triplex than the unmodified one. This type of modification may be useful for triplex-based approaches in a cellular context by decreasing both the active concentration of TFO and the non-triplex-related effects.

Experimental

Unmodified oligonucleotides and plasmids

The unmodified oligodeoxynucleotides (the two complementary 29-mer and 15G) were obtained from Eurogentec (Belgium). The oligonucleotide acridine-conjugate (Acr-15G) was purchased from Quantum Appligene (France). The pLTR plasmid was obtained by inserting a 1440 bp fragment of the viral HIV-1 DNA containing the 16-bp oligopyrimidine·oligopurine target sequence in pBR328.[23]

Preparation of the modified nucleoside 7-chloro-7-deaza-2′-deoxyguanosine

This compound was obtained using a procedure adapted from the literature.[20,21]

White crystals. CCM: $R_f = 0.37$ using a mixture of dichloromethane and methanol (80:20, v/v) as eluent.

^{1}H NMR. (DMSO-d_6): δ, 10.5 (s, 1H, N–H), 7.05 (s, 1H, H_6), 6.38 (bs, 2H, NH_2), 6.30 (dd, $J = 5.9$ Hz, $J = 8.20$ Hz, 1H, $H_{1'}$), 5.2 (d, 1H, $OH_{3'}$), 4.9 (t, 1H, $OH_{5'}$), 4.3 (m, 1H, $H_{3'}$), 3.76 (m, 1H, $H_{4'}$), 3.5 (m, 2H, $H_{5'}$, $H_{5''}$), 2.3 (m, 1H, $H_{2'}$), 2.06 (m, 1H, $H_{2''}$). Mass analysis. I.S. polarity positive. Calculated for $C_{11}H_{13}ClN_4O_4$: M + H = 301.07 and 303.07. Found: 301.4 and 303.0.

Preparation of the phosphoramidite derivative of the modified nucleoside 7-chloro-7-deaza-2′-deoxyguanosine

After protection of the exocyclic amino function of the nucleic base with the isobutyryl group and protection of the 5′-hydroxyl function with dimethoxytrityl group, the phosphoramidite derivative was obtained following a procedure adapted from the literature.[22] Pale yellow foam. CCM: $R_f = 0.30$ using a mixture of dichloromethane, ethyl acetate and triethylamine as eluent (90:10:1, v/v/v). ^{31}P NMR. ($CDCl_3$). (ref $OP(OMe)_3$), δ ppm: 143.06 and 143.74.

Preparation of oligonucleotides (1–5)

Oligonucleotides were synthesized on a 1 μmol scale on an Expedite Nucleic Acid Synthesizer using standard β-cyanoethyl phosphoramidite chemistry.[24] Reagents were prepared as described in the user manual and a standard concentration of a phosphoramidite solution was used for all the phosphoramidites including that of the modified nucleoside. Coupling efficiency was estimated from trityl assays and were >95% per step for the modified nucleoside. After the chain assembly (trityl-on mode) oligomers **1**–**3** were removed from the solid support and deprotected by concentrated aqueous ammonia treatment (28% aq NH_3) (2 mL) at 55 °C for 15 h. The ammonia solution was then removed by evaporation.

In the case of the acridine–oligonucleotide conjugates **4** and **5**, at the end of the chain assembly an additional detritylation step was performed and the acridine was added manually via its phosphoramidite derivative.[25] Acridine–oligonucleotide conjugates **4** and **5** were removed from the solid support and deprotected by treatment with 0.4 M NaOH in a MeOH/H_2O (50:50, v/v) solution as follows: 1 ml of the deprotecting solution was added on the support. After 30 min, the solution was removed and another mL of the deprotecting solution was added. After another 30 min of incubation, the solution was removed, pooled with the first fraction obtained and the resulting solution was allowed to stand in the dark at room temperature. After 48 h, the pH of the solution was adjusted to 5–6 with acetic acid (18 M), the methanol was removed by evaporation and the oligonucleotide solution was filtered off using a 0.45 μM filter. NaOH was used instead of concentrated aqueous ammonia in order to prevent the cleavage between the C(9) atom of the acridine ring and the N atom of the linker.[25] Oligonucleotides **1**–**3** were purified on a reversed-phase Lichrocart column (250×10 mm) packed with Lichrosorb RP 18 (10 μm) using a linear gradient of acetonitrile from 20 to 32% over 30 min, in 0.1 M aqueous triethylammonium acetate buffer, pH 7, with a flow rate of 4 mL/min and detection at λ = 260 nm. After removal of acetonitrile by evaporation and lyophilization, oligonucleotides **1**–**3** were detritylated by aqueous acid acetic treatment (80%) for 20 min at room temperature. After purification the purity of all the oligodeoxyribonucleotides (**1**–**5**) described was verified by reversed-phase analysis using a Lichrocart system (125×4 mm) packed with Lichrospher RP 18 (5 μm) from Merck eluted with a linear gradient of acetonitrile from 0 to 27.5% over 30 min, in a 0.1 M aqueous triethylammonium acetate buffer, pH 7, with a flow rate of 1 ml/min. The retention times (Rt) of oligonucleotides were as follows: $Rt_1 = 15$ min, 14 s (broad peak). $Rt_2 = 16$ min, 9 s, $Rt_3 = 16$ min 51 s (40%) and 22 min, 9 sec (60%), $Rt_4 = 20$ min, 23 s and $Rt_5 = 21$ min, 8 s. Oligonucleotide **3** (containing a run of five modified G*) gave two peaks in almost equal quantities on both analytical and preparative reversed-phase columns. The products corresponding to each peak were collected separately and again sujected to HPLC analysis using different systems. On the above analytical RP C18 column, each compound gave once again two peaks with retention times identical to those previously mentioned above. The same phenomenom was observed by ion exchange analysis on a DEAE column (8 μm, 100×10 mm, Waters) with a linear gradient (0.375–0.9 M) of NaCl in 25 mM Tris/HCl buffer (pH 8), containing 10% CH_3CN, over 35 min. Two peaks were obtained Rt = 19 min, 8 sec (45%) and 25 min, 11 sec (55%). Analysis by 20% polyacrylamide gel electrophoresis using denaturing conditions (7 M urea) indicates (Fig. 2) that none of the modified oligodeoxyribonucleotides forms aggregates as opposed to the unmodified sequence **6** containing a run of six 2′-deoxyguanosines used as reference in our experiments. Migration was similar for the three modified oligodeoxyribonucleotides **1**–**3** although oligonucleotide **1** gave a less resolved band than did oligonucleotides **2** and **3**. In the case of the oligodeoxyribonucleotide **3** the same mobility was observed for the two products recovered during purification by reversed-phase chromatography.

Full deprotection and nucleoside composition of the modified oligonucleotides **2**, **4** and **5** were ascertained by nuclease degradation. An aliquot of oligonucleotide was digested with snake venom phosphodiesterase (Pharmacia Biotech) and alkaline phosphatase (Boehringer) in 0.1 M Tris/HCl, pH 8.2, for 3 h at 37 °C. After inactivation of the enzyme at 90 °C for 2 min, the digestion products were analyzed by reversed-phase chromatography using a Lichrocart system (125×4 mm) packed with Nucleosil 100-5 C18 from Macheray-Nagel equilibrated with a 0.1 M aqueous triethylammonium acetate buffer, pH 7. The column was eluted at a flow rate of 1 ml/min with 0.1 M aqueous triethylammonium acetate buffer, pH 7, for 10 min and then with a linear gradient of 0–24% of acetonitrile in a 0.1 M aqueous triethylammonium acetate buffer, pH 7, for 30 min and with a linear gradient of 24 to 56% of acetonitrile in a 0.1 M aqueous triethylammonium acetate buffer, pH 7, for 20 min. Detection was performed at 260 nm. Oligonucleotide **2** was totally degraded to nucleosides. Four peaks were

obtained. Comparison with natural and modified nucleoside samples allowed us to identify the different peaks, of which three showed retention times corresponding to $d^{5Me-}C$ (Rt = 7 min), dT (Rt = 10 min, 48 s), and dG (Rt = 12 min, 48 s), respectively. The presence of the modified $d^{7Cl-}G$ was verified at Rt = 22 min, 32 sec. The peak area ratio between dG and $d^{7Cl-}G$ confirms the presence of three modified dG. In the case of the acridine–oligonucleotide conjugate **4** and **5** in addition to the above mentioned nucleosides, the presence of the acridine-linker derivative was observed at Rt = 52 min. As previously reported, prolongated treatment at pH 8.2 produces a side product with a lower retention time than that of the acridine-linker derivative. This product, 6-chloro-2-methoxy-9-acridone, comes from the acridine-linker derivative by cleavage of the C(9)-N linkage.

Mass analysis. I.S. polarity negative. Calculated for **1:** $C_{152}H_{191}Cl_2N_{47}O_{96}P_{14}$, M = 4716.99 Da. Found: 4716.26 ± 1.43 Da. Calculated for **2**: $C_{153}H_{191}Cl_3N_{46}O_{96}P_{14}$, M = 4750.44 Da. Found: 4750.17 ± 0.31 Da. Calculated for **3**: $C_{155}H_{191}Cl_5N_{44}O_{96}P_{14}$, M = 4817.36 Da. Found for compound corresponding to the first peak 4816.52 ± 1.58. Found for compound corresponding to the second peak 4817.48 ± 0.77 Da. Calculated for **5**: $C_{173}H_{213}Cl_4N_{48}O_{100}P_{15}$, M = 5171.28 Da. Found: 5170.90 ± 0.21 Da.

T_m measurements by UV absorption experiments

Experiments were carried out in a 10 mM sodium cacodylate buffer (pH 6.5) containing 10 mM $MgCl_2$ and either 50 mM NaCl or 150 mM KCl, as indicated. A cacodylate buffer was chosen due to its limited dependence of pH on temperature. UV melting curves were obtained using a Uvikon 940 spectrophotometer interfaced to an IBM computer. The temperature of the cell holders was regulated by a Haake P2 circulating bath. The temperature was regulated at a rate of 0.2 °C/min by a Haake PG 20 thermoprogrammer. To monitor triplex-to-duplex transition, the samples were first heated and then cooled. Oligonucleotide concentrations were 1 μM of the duplex target D (with a 1/1.2 ratio of purine/pyrimidine-containing strands), and 1.5 μM of the third strand (see sequences in Fig. 1). The absorbance at 260 and 540 nm was recorded every 5 min. Corrections for spectrophotometric instability were made by substracting the absorbance at 540 nm from that at 260 nm. Cooling and heating curves were reversible. The uncertainty in the T_m values reported is estimed at ±1 °C.

Triplex formation analysis by gel mobility shift assay

For gel shift assays we used two DNA duplexes containing either the PPT/HIV-1 oligopyrimidine•oligopurine sequence (29-bp long, with the following sequence of the oligopurine-containing strand: 5′ CCACTTTTT AAAAGAAAAGGGGGGA CTGG 3′) or the mutated version (34-bp long, with the following sequence of the oligopurine-containing strand: 5′ CACATTTTAT AAAAGAAAAaGGaGGA CTGGAAGG 3′). The pyrimidine-rich strand of this duplex was 5′-end labeled by treatment with T4 polynucleotide kinase and [γ^{32}P] ATP under standard conditions. Duplex (50 nM) was formed by annealing the pyrimidine-rich sequence with the complementary sequence at a ratio of 1/1.2. Triplex formation was conducted at 25 °C overnight in a final volume of 10 μl. The standard reaction buffer was 50 mM MES, pH6, 50 mM NaCl, 10 mM $MgCl_2$, 10% sucrose. The concentration of labeled duplex was kept constant (50 nM) while the concentration of the TFOs was varied (0.1–10 μM) (see Fig. 3). Triplexes were analyzed in a 15% nondenaturing polyacrylamide gel prepared in 50 mM MES, pH 6, containing 10 mM $MgCl_2$. The running temperature was approximately 25 °C. Dried gels were quantified using a phosphorimager.

Inhibition of DraI cleavage by TFOs directed against the PPT/HIV-1 sequence

The restriction enzyme DraI was obtained from Boehringer. Enzymatic assays (1u/λ DraI) were performed at 37 °C in a 50 mM Hepes buffer (pH 7.2) containing 10 mM $MgCl_2$, 50 mM NaCl and 0.5 mM spermine. The pLTR plasmid contained 4 DraI cleavage sites (TTT↓AAA) one of which overlapped three base pairs of the PPT triplex site. After incubation, the enzymatic reaction was stopped by adding EDTA (25 mM). The circularized plasmid pLTR (2γ/10λ) was incubated in the absence (lane (−)) or in the presence of increasing concentrations of 15-mer oligonucleotides as indicated near the gels (Fig. 5).

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