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The high stability of the triple helices formed between short purine oligonucleotides and SIV/HIV-2 vpx genes is determined by the targeted DNA structure

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ABSTRACT

In our previous works we have shown that the oligonucleotides 5′-GGGGAGGGGGAGG-3′ and 5′-GGAGGGGGAGGGG-3′ give very stable and specific triplexes with their target double stranded DNAs [Svinarchuk, F., Bertrand, J.-R. and Malvy, C. (1994) *Nucleic Acids Res.*, 22, 3742–3747; Svinarchuk, F., Paoletti, J. and Malvy, C. (1995) *J. Biol. Chem.*, 270, 14 068–14 071]. The target for the invariable part of these oligonucleotides, 5′-GGAGGGGGAGG-3′, is found in a highly conserved 20 bp long purine/pyrimidine tract of the vpx gene of the SIV and HIV-2 viruses and could be a target for oligonucleotide directed antivirus therapy. Here we report on the ability of four purine oligonucleotides with different lengths (11-, 14-, 17- and 20-mer) to form triplexes with the purine/pyrimidine stretch of the vpx gene. Triplex formation was tested by joint dimethyl sulfate (DMS) footprint, gel-retardation assay, circular dichroism (CD) and UV-melting studies. Dimethyl sulfate footprint studies revealed the antiparallel orientation of the third strand to the purine strand of the Watson–Crick duplex. However, the protection of the guanines at the ends of the target sequence decreased as the length of the third strand oligonucleotide increased. Melting temperature studies provided profiles with only one transition for all of the triplexes. The melting temperatures of the triplexes were found to be the same as for the targeted duplex in the case of the 11- and 14-mer third strands while for the 17- and 20-mer third strands the melting temperature of the triplexes were correspondingly 4 and 8°C higher than for the duplex. Heating and cooling melting curves were reversible for all of the tested triplexes except one with the 20-mer third strand oligonucleotide. Circular dichroism spectra showed the ability of the target DNA to adopt an A-like DNA conformation. Upon triplex formation the A-DNA form becomes even more pronounced. This effect depends on the length of the third strand oligonucleotide: the CD spectrum shows a 'classical' A-DNA shape with the 20-mer. This is not observed with the purine/pyrimidine stretch of the HIV-1 DNA which keeps a B-like spectrum even after triplex formation. We suggest, that an A-like duplex DNA is required for the formation of a stable DNA purine(purine–pyrimidine) triplex.

INTRODUCTION

Homopurine–homopyrimidine regions in DNA have attracted a great deal of attention in connection with their possible role in gene regulation in eukaryotes (1,2). These regions raise the possibility of manipulating gene expression and virus propagation through artificial triple helix formation (3,4).

In our previous work (5), we have shown that the oligonucleotide 5′-GGGGAGGGGGAGG-3′ binds to its target duplex sequence in the presence of 50 mM Na^+ or K^+, 10 mM $MgCl_2$ and 20 mM Tris-acetate, pH 7.5. This oligonucleotide is bound in an antiparallel orientation with respect to the homopurine sequence. As was shown by a co-migration assay, the triplex is stable up to 65°C. At 37°C triplex formation was practically irreversible: after 24 h of co-migration assay there was no traces of triplex dissociation. The sequence with a similar composition but reversed sequence, 5′-GGAGGGGGAGGGG-3′, has been shown to stabilize its target double stranded DNA (6). The target for the invariable part of these oligonucleotides, 5′-GGAGGGGGAGG-3′, is found in the provirus DNA of SIV and HIV-2 within a highly conserved 20 bp long purine/pyrimidine tract of the vpx gene (7). This sequence thus can be considered as a potential target for oligonucleotide directed antivirus therapy.

Since the data concerning the influence of the length of the triplex-forming oligonucleotides (TFO) on the triplex stability are still contradictory (5,6,8) we wished to assess the ability of oligonucleotides with different length (11-, 14-, 17- and 20 mer)

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to form triplexes with the purine/pyrimidine stretch of the vpx gene. Triplex formation was tested by dimethyl sulfate (DMS) footprint, gel-retardation assay, circular dichroism (CD) and UV-melting studies. It was found that the four tested purine oligonucleotides yield triplexes presenting the same or even higher T_m than the target 20 bp long DNA. These types of triplexes will be considered as 'stable' in the present work. We suggest, that an A-like form of duplex DNA is a prerequisite for the formation of the stable DNA purine (purine–pyrimidine) triplexes with an antiparallel orientation of the third strand relatively to the purine strand of the Watson–Crick duplex.

MATERIALS AND METHODS

Oligonucleotide preparation

Oligonucleotides were synthesized on an Applied Biosystems 391A DNA synthesizer using the solid phase phosphoramidite procedure. They were precipitated with 10 vol of 3% solution of $LiClO_4$ in acetone and the pellets were washed with acetone, dried and dissolved in water. Concentrations were determined spectrophotometrically, using extinction coefficients calculated from extinction coefficients for nucleotides and dinucleotide phosphates by the equation given in ref. 9. For the gel-retardation assay and CD studies the oligonucleotides were purified by electrophoresis in a 20% polyacrylamide denaturing gel. The target duplex DNA for CD studies was prepared by electrophoresis in a 10% non-denaturating gel after heating of an equimolar mixture of the complementary oligonucleotides up to 80°C and then a slow cooling to room temperature. After electrophoresis the oligonucleotides were eluted from the gel in 1 ml of 0.2 M $LiClO_4$ solution during 12 h at 37°C followed by a precipitation with 10 vol of acetone. The 5′ end of the pyrimidine strand of the duplex was radiolabelled with [γ-^{32}P]ATP (Amersham) by T4 polynucleotide kinase (New England Biolabs) as per manufacturer's instructions. The oligonucleotide was labelled at specific activity of 20 Ci/mmol.

Plasmid construction

The plasmid pR1 containing the SIVMM25 vpx gene was made by inserting the *Bam*HI–*Hin*dIII fragment (440 bp) of pTG 651 (Transgene) into the *Bam*HI–*Hin*dIII sites of the vector pBluescript II (Stratagene). All plasmids were grown in bacterial strain XL Blue 1 (Stratagene) and purified by CsCl gradient methods (10).

Gel-retardation assay

For the gel-retardation assay the 20 bp double stranded DNA (50 pM) was mixed with the third strand DNA in a proportion indicated in the figure legends. The buffer contained 10 mM $MgAc_2$, 50 mM NaAc and 20 mM Tris-Ac, pH 7.5. After 60 min incubation of the mixture at 37°C and cooling to room temperature the triple helix formation was monitored by 10% polyacrylamide gel electrophoresis in the same buffer. Electrophoretically separated duplex and triplex DNA were visualized by autoradiography.

DMS footprint

To prepare a DNA fragment for modification by DMS the pR1 plasmid was cut with *Bst*BI restriction enzyme, 3′ labelled with Klenow fragment of DNA polymerase I and digested with *Hin*dIII restriction enzyme. A smaller labelled fragment (~0.5 pM) was dissolved in 20 µl of the buffer: 50 mM MOPS, pH 7.2, 50 mM NaCl and 10 mM $MgCl_2$. Then 50 pM of the triplex forming oligonucleotide was added. The mixture was incubated for 1 h at 37°C. Then 2 µl of 5% DMS was added and the reaction was performed for 3 min at 25°C. The reaction was stopped by the addition of a 5 µl solution containing 10% mercaptoethanol, 1 mM EDTA and 0.1 M Na acetate. After double precipitation in ethanol the samples were treated with 50 µl of 10% piperidine at 95°C for 20 min and the cleavage products were separated in 6% polyacrylamide denaturing gel.

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Duplex VPX:          5'-CCTCCTCCTCCTCCCCCTCC-3'
                     3'-GGAGGAGGAGGAGGGGGAGG-5'

Third strands VPX:
        VPX11                      5'-GGAGGGGGAGG-3'
        VPX14                   5'-GGAGGAGGGGGAGG-3'
        VPX17                5'-GGAGGAGGAGGGGGAGG-3'
        VPX20             5'-GGAGGAGGAGGAGGGGGAGG-3'


Duplex C1:                   5'-TCCCCCCTTTTCTTTT-3'
                             3'-AGGGGGGAAAAGAAAA-5'

Third strand C1:             5'-AGGGGGGAAAAGAAAA-3'


Duplex C2:           5'-CCTCCTCCTCCTCCTCTCCT-3'
                     3'-GGAGGAGGAGGAGGAGAGGA-5'

Third strand C2:     5'-GGAGGAGGAGGAGGAGAGGA-3'
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Figure 1. Duplex targets and triplex-forming oligonucleotides. The duplex VPX corresponds to a 20 bp polypurine/polypyrimidine region of the SIV and HIV-2 vpx gene. The duplex C1 corresponds to the polypurine/polypyrimidine region of HIV-1 and the duplex C2 corresponds to the polypurine/polypyrimidine region of the human integrin (CD18) gene. The third strand oligonucleotides were designed to bind in an antiparallel orientation when compared to the purine strand of the duplexes.

UV spectroscopic temperature dependent melting studies

Absorbance of the oligonucleotide mixtures was measured at 258 nm as a function of temperature with an Uvicon 941 spectrophotometer equipped with a Bioblock Ministat cryothermostat and a Huber PD415 temperature programmer through software developed for melting temperature (T_m) recording. The rate of temperature increase was 0.5°C/min. The buffer contained 10 mM $MgAc_2$, 50 mM Na-acetate and 20 mM Tris–HCl, pH 7.5. Concentration of each oligonucleotide was 1.0 µM. Before melting studies all samples were heated to 80°C for 15 min and then allowed to return slowly to room temperature.

CD studies

CD spectra between 205 and 320 nm were recorded using a Jobin-Yvon Mark IV high-sensitivity dichrograph linked to a IBM PC compatible computer, using a cell of 0.5 cm path length. The temperature of the samples was kept with the accuracy of ± 0.1°C during each spectral measurement using a thermostat controlled cell holder linked to a Cole-Palmer thermistor. The concentration

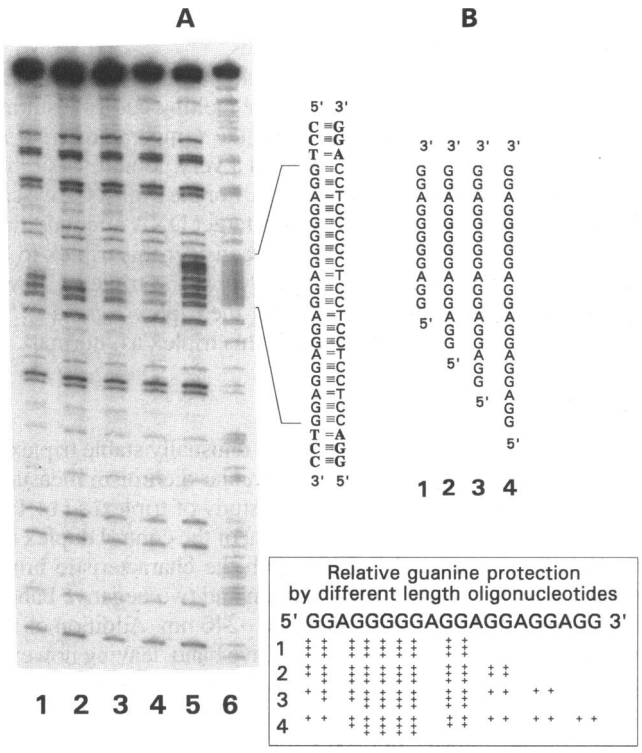

Figure 2. Dimethyl sulfate footprinting experiments carried out with different length third strand oligonucleotides. (**A**) Autoradiogram of a 6% polyacrylamide sequencing gel showing the results of DMS footprinting experiments carried out with third strand oligonucleotides of increasing length: line 1, 11-mer; line 2, 14-mer; line 3, 17-mer; line 4, 20-mer; line 5, control oligonucleotide 5′-GAGGCGGCAGGGCGAGAGGC-3′; line 6, A + G reaction. (**B**) Sequences of the TFO used in lines 1–4. The inserted Table shows the relative protection of the guanines by different length oligonucleotides.

of each oligonucleotide was ~1 μM and the buffer contained 10 mM $MgAc_2$, 50 mM Na-acetate and 20 mM Tris–HCl, pH 7.5.

RESULTS

DMS footprint

The oligonucleotides which were used in the present experiments are listed in Figure 1. For the oligonucleotides targeted to the vpx gene, the triplex formation was monitored by DMS footprint (Fig. 2). Dimethyl sulfate modifies the N7 position of guanines leading to chain cleavage after treatment with piperidine. This chemical does not react with the N7 position of the purines of the double-stranded DNA within a purine–purine–pyrimidine triplex because they are protected by Hoogsteen base pairing (11). As shown in Figure 2 the guanines located within the target sites for all tested oligonucleotides: VPX11, VPX14, VPX17 and VPX20, are less reactive with DMS than guanines external to the sites, indicating triplex formation under experimental conditions. This experiment shows the antiparallel orientation of the third strand oligonucleotides: in the case of the parallel orientation the fifth G from the 5′ end of the target would not be protected. In the experiment with the oligonucleotide 5′-GGAGGGGGAGGAG-GAGGAGG-3′ designed to form a triplex in the parallel orientation this fifth G was not protected at all, indicating that in

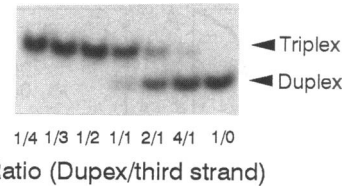

Figure 3. Gel-retardation assay with the VPX duplex DNA and the third strand oligonucleotide VPX20. Sequences of the oligonucleotides are given in Figure 1.

fact even this oligonucleotide binds in the antiparallel orientation (data not shown). Curiously, it was observed that the protection becomes weaker with the increase in oligonucleotide length.

Gel retardation assay

The gel retardation assay reported in Figure 3 shows that by increasing the ratio duplex DNA:third strand oligonucleotide up to 1:1 it remains only a light band with a mobility corresponding to double stranded DNA, while a slower migrating band appears. The disappearance of the double stranded DNA band at the ratio 1:1 duplex:third strand DNA indicates that the low migrating band corresponds to a triple helical structure.

UV melting studies

One could expect, that a lower degree of guanine protection in DMS footprint experiments would correspond to less stable triplexes, but this is not the case. We monitored the stability of both the targeted duplex and the resulting triplex by UV spectroscopic temperature dependent melting studies. As shown in Figure 4 there is only one transition for both the duplex and the triplexes VPX. The triplexes obtained with the VPX11 and VPX14 third strands present the same T_m as that of the targeted duplex, while the triplexes formed with VPX17 and VPX20 show T_m which are correspondingly 3 and 8°C higher than the duplex T_m (Table 1).

Table 1. The T_m of the targeted duplexes and the triplexes with the corresponding third strands

Duplexes	VPX				C1	C2
T_m (°C) of duplexes	72.1				57.0	70.0
Third strand oligonucleotides	VPX11	VPX14	VPX17	VPX20	C1	C2
T_m (°C) of triplexes	72.1	72.5	75.9	79.8	40.0	74.2

Sequences of the oligonucleotides are given in Figure 1. Melting temperatures are calculated from the heating curves at 1 μM concentration of each oligonucleotide. Data are based on the analysis of three melting curves for each T_m. Accuracy of T_m determination is 0.5°C.

The triplexes formed with the oligonucleotides VPX11, VPX14 and VPX17 displayed a very good reversibility of the melting profiles with a rate of heating/cooling of 0.5°C/min, which indicates a high rate of triplex formation. This is a higher rate than those reported for other triplexes (12,13). For example, Rougee *et al.* (13) have described melting curves with an hysteresis profile using a rate of heating/cooling of 0.12°C/min.

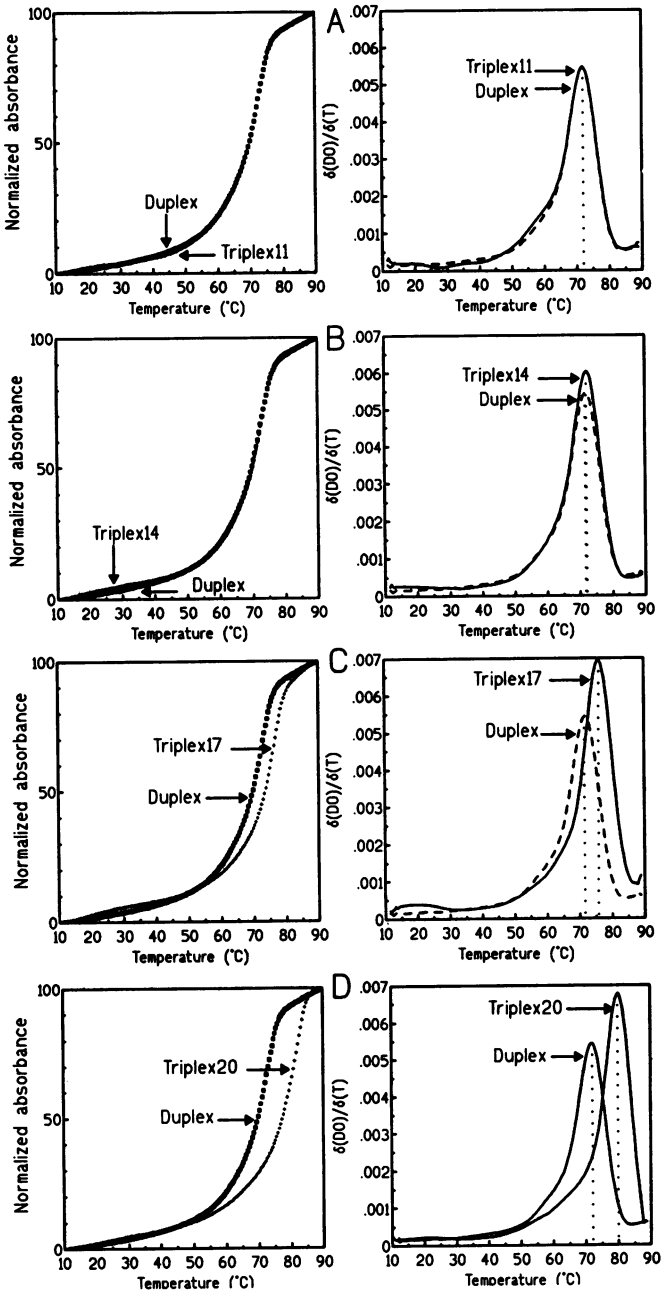

Figure 4. Heating T_m curves and their derivatives of the equimolar mixture of the VPX duplex DNA and the third strand oligonucleotides of different length. Sequences of the oligonucleotides are given in Figure 1.

In contrast, the melting curves obtained for the triplex with the third strand oligonucleotide VPX20 did not reflect reversibility even with a rate of heating/cooling of 0.05°C/min, at 1 μM concentration of each oligonucleotide. By varying the rate of temperature changes we have shown, that thermal renaturation of the triplex is a quick process (there was no difference in the shape of the cooling curves with a cooling rate either 0.5 or 0.05°C/min). At the same time the heating curves were 'rate-dependent' and they did not fit the cooling curve even with the rate of heating 0.05°C/min. We suggest that before melting the triplex structure passes over an energetic barrier or undergoes a slow rearrangement step. We are currently studying the thermodynamic and kinetic parameters of the stable triplexes.

For the triplexes formed between the duplex and the third strand oligonucleotides C1 and C2 (Fig. 1) the melting curves resulting from either increasing or decreasing the temperature (rate of heating/cooling of 0.5°C/min) showed a very good reversibility of the melting profiles (data not shown). For the triplex C2 we can also note an increase in stability of the target DNA after addition of the third strand (Table 1). Thus we consider this triplex to be classified as 'stable'. The melting curve of the triplex C1 was biphasic giving the values of 40°C for the triplex T_m and 57°C for the T_m of the duplex. We classify this triplex as 'normal'.

CD studies

To clarify the structural basis for these unusually stable triplexes we performed CD studies (Fig. 5). Circular dichroism measurements proved extremely useful in the study of triplexes (14,15). As one can see (Fig. 5C) the CD spectra of the control duplex C1 corresponds to a normal B DNA with the characteristic broad positive band between 250 and 280 nm and two negative bands: a small one ~210 nm and another one ~240 nm. Addition of the third strand leads to a more sharp positive band, leaving however the shape of the spectrum mainly B-form. A different behavior is found for the stable PX and C2 triplexes (Fig. 5A and B). The CD spectra of the starting duplexes reflect a high content of the A-form, mostly defined by more intensive bands at ~260 (positive) and 210 nm (negative). Addition of the third strand VPX20 or C2 to these duplexes strongly shifts the spectra to the typical A-form well depicted by a strong narrow positive signal at 260 nm ($\Delta\varepsilon$ is >4) and two negative signals at 240 and 210 nm (16,17). As shown in the Figure 5A this shift effect for the VPX triplexes decreases as the length of the third strand oligonucleotide is shortened. Heating of the samples to 50°C does not change the CD spectra of the VPX and C2 triplexes, confirming the stability of the triplex structure at this temperature (Table 1). At the same time the CD spectrum of the C1 triplex at 50°C is equivalent to the sum of the duplex and the third strand oligonucleotide CD spectra (data not shown), indicating the loss of the triplex structure.

Mg^{+2} (or other divalent cation, we did not test this possibility) is absolutely required for triplex formation (5,6,18–20). Our CD measurements confirm this fact. Variation of the Mg^{+2} concentration from 0 to 10 mM does not change the spectrum of the VPX duplex. However, addition of the third strand VPX20 to the duplex changes the spectrum to the A-form only in the presence of 10 mM Mg^{+2}. Upon addition of 10 mM EDTA the spectrum is immediately changed to the sum of the duplex and the third strand spectra.

DISCUSSION

We have found in a previous work, that the oligonucleotide 5′-GGGGAGGGGGAGG-3′ gives a very particular triplex structure with its target sequence in the *c-pim-1* protooncogene promoter (5). The *in vitro* parameters of the triplex formation are very different from those measured by other authors (3,21) as: (i) the triplex is stable at 65°C in a co-migration assay; (ii) a single mismatch in the target sequence completely abolishes triplex formation as judged by the co-migration assay; (iii) the value of the dissociation constant can be estimated to be $<1.5 \times 10^{-9}$ M; 4) once formed, the triplex is practically irreversible at 37°C: after

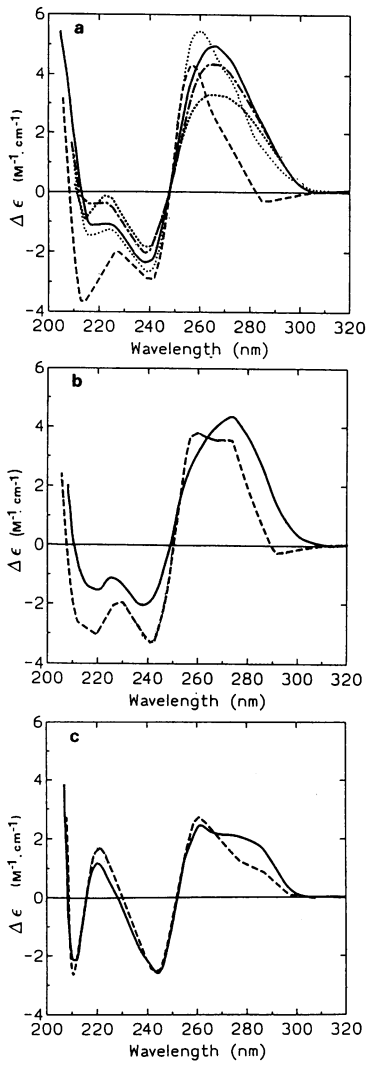

Figure 5. CD spectra of the duplexes (—) and triplexes with the corresponding third strands: (**a**) VPX11 (·-·), VPX14 (--), VPX17 (···), VPX20 (---); (**b**) C2 (---); and (**c**) C1 (---). Sequences of the oligonucleotides are given in Figure 1. Ellipticity is expressed per mol of nucleotide.

24 h of co-migration assay, there is no trace of triplex dissociation. We have also shown for the inverted sequence, 5′-GGAGGGG-GAGGGG-3′, that the triplex formation stabilizes the double stranded targeted DNA (6). The 11-mer 'core' sequence of these two oligonucleotides, 5′-GGAGGGGGAGG-3′, is found in the vpx gene of the SIV and HIV-2 and it was tempting to check its potential to form stable triplex in the frame of gene-targeted antivirus drug studies. The sequence 5′-GGAGGGGGAGG-3′ is located inside the long purine stretch 5′-GGAGGGGGAGGAG-GAGGAGG-3′ at the 3′ end of the vpx gene, and this stretch is invariant among many isolates of SIV and HIV-2.

In order to estimate an optimum length of the TFO we tested the 11-, 14-, 17- and 20-mer (Fig. 1). Our DMS footprint experiments showed that all of them are able to protect the guanine residues in the target sequence to different degrees, indicating triplex formation under experimental conditions. Unexpectedly, the DMS footprint experiments determined that the level of guanines protection decreased together with the increasing of the TFO length, this effect being more pronounced for guanines located at the 3′ end of the target sequence. At the same time T_m studies show that the 17- and 20-mer TFO are forming triplexes with T_m which are correspondingly 3 and 8°C higher than for the duplex alone which is in agreement with our previous findings with the oligonucleotide 5′-GGAGGGGGAGGGG-3′ (6). Particularly surprising is the fact that the triple helix stability increases with the length of the third strand oligonucleotides whereas the opposite is true for the level of guanine protection. It is thus tempting to suggest that the main factors contributing to the stability of the stable triplexes is not the reverse Hoogsteen base pairing but rather a particular DNA conformation allowing for example better stacking interaction between the bases of the third strand. It is also possible that due to the high G content the extremities of the longest oligonucleotides adopt some folded structure.

On another hand the CD spectra of the control duplex C1 were typical for DNA structures still having a high B-DNA content (16,17), while the spectra of the duplexes VPX and C2 were more consistent with the A-form (15–17). We found that addition of the third strand to the C1 duplex resulted in spectra still keeping the main features of the B-form DNA. In contrast, addition of the third strands to the VPX and C2 duplexes made the shape of the triplex CD spectra more typical for A-form DNA. Thus, the two duplexes providing the more stable triplexes displayed A-like CD spectra, these became even more typical for A-form DNA upon triplex formation. This contrasted with the behavior of the C1 duplex and triplex which both showed B-like spectra and whose T_m values (57 and 40°C respectively) indicated less stable triplex.

Is the A-form of duplex DNA an obligatory condition for the formation of a stable triplex? Firstly, in examples where very stable intermolecular pyrimidine(purine–pyrimidine) triplexes occur it has been shown by Fourier transform infrared and NMR spectroscopies, that the triplex formation between a TFO RNA and a duplex DNA is associated with the conversion of the duplex partner from a B-form to a form which contains partly A-form sugars (22). Second, Cheng and Pettitt (23) by a theoretical approach have found that in order to accommodate a third strand in triplex formation, the backbone of the B-DNA duplex has to be adjusted into A-like DNA.

Present data suggest that a duplex DNA which has already shifted into an A-form (or in a mixture of conformations highly populated in the A-form), will need less energy to adopt a stable purine(purine-pyrimidine) triplex organization than a duplex which has to undergo a full B-DNA to A-DNA transition. The depth of the major groove seems to be determinant to accommodate the reverse Hoogsteen base pairing with the third strand (23). The role of base stacking is also probably significant for the triplex stabilization, but the balance of energetics needs further discussion in terms of cation (Mg^{+2}) and probably solvent molecules (23).

We hope that this work will stimulate modeling studies of triplex structures and will help in a search of other similar sequences which could be good targets for gene-targeted therapy.

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