Pyrimidine phosphorothioate oligonucleotides form triple-stranded helices and promote transcription inhibition

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ABSTRACT

The ability of phosphorothioate (POS) oligonucleotides to recognise and bind to homopurine – homopyrimidine DNA double-stranded sites via triple helix formation has been investigated. It has been found that the homologous pyrimidine POS sequences Y11-S_i (i = 0, 1, 2, 3, 4, 10), which have been obtained by an increasing sulphur substitution in the sugar – phosphate backbone of d(CTTCCTCCTCT) (Y11), and the target hairpin duplex d(GAAGGAGGAGA-T₄-TCTCCTCCTTC) (h26) can form stable triple helices, as indicated by PAGE, CD and UV melting experiments. The thermal stability of the triple helices depends on the number of POS linkages in the third Y11 strand, varying from 48°C (Y11, with only phosphate groups, PO₂) to 31°C (Y11-S₁₀ containing exclusively thioate groups). On average, a *T_m* depression of about 2°C per POS linkage introduced in Y11 was observed. CD data indicate that the sulphurization of the third strand results in minimal changes of triple-stranded structures. The energetics of the triplex-to-hairpin plus single-strand transition has been determined by van't Hoff analyses of the melting curves. In free energy terms, the POS triplexes h26·Y11-S_i are less stable than the normal PO₂ h26·Y11 triplex by values between 2.7 and 5.4 kcal/mol, depending on the number of POS linkages contained in the third strand. Phosphorothioate oligonucleotides being resistant towards several nucleases offer an interesting choice as *gene blockers* in antisense strategy. Thus, their ability to inhibit transcription via triple helix formation has been examined *in vitro*. We found that triplex-forming POS oligonucleotides of 20 bases in length (with a cytosine contents of 45%), containing either 10% or 26% thioate groups, strongly repress the transcription activity of the bacteriophage T7 RNA polymerase at pH 6.9, when used in excess compared to the target (mol oligo/mol template = 125). The here reported data are useful for designing phosphorothioate oligonucleotides targeted to genomic DNA in antise strategy.

INTRODUCTION

The sequence-specific recognition of double-stranded DNA by pyrimidine oligonucleotides through a mechanism based on triple helix formation has been demonstrated (1–3). The pyrimidine oligonucleotides bind to the major groove of homopurine – homopyrimidine (R·Y) double-stranded DNA with a parallel orientation with respect to the purine strand of the Watson–Crick target helix, generating a pyrimidine·purine·pyrimidine (Y·R·Y) triplex (1,4–6). Sequence specificity is due to T and protonated C of the third strand recognising AT and GC base pairs of the target duplex respectively, forming isomorphous T·AT and C⁺·GC triads with Hoogsteen configuration (5–10). The thermodynamic stability of Y·R·Y triple helices depend on a number of factors including length, sequence composition, pH and ionic strength (11–17). Another triplex motif consisting of two antiparallel purine and one pyrimidine strands has lately been described (18–23).

Recently, interest in this unusual DNA structure has grown, as triplex-forming oligonucleotides targeted to a promoter region of a gene may be used to artificially inhibit its transcription (24–26). Experiments conducted *in vitro* (25–28) and *in vivo* (29–31) have demonstrated the potential of this strategy, as an alternative to the more common antisense bio-technology (32–33). However, the therapeutic value of normal oligonucleotides is limited by the fact that these compounds are easily degraded by the nucleases present in the cells and serum (34–37). To solve this problem several important modifications of the phosphate backbone have been proposed: the substitution of phosphate groups with phosphorothioates (36,38–40), phosphorodithioates (41–43), methylphosphonates (29,44–45) and riboacetals (46). In addition, a number of base and backbone modifications have been reported to improve triplex formation by pyrimidine oligonucleotides (13,47,48). While the physicochemical properties of double-stranded oligo- and polynucleotides containing phosphorothioate linkages have been determined (36, 49–53), not much is known about the ability of phosphorothioate oligonucleotides to bind double-stranded DNA via triple helix formation (52–54). According to recent data it seems that purine-rich oligonucleotides containing exclusively thioate groups bind duplex DNA with a greater affinity than all-thioate pyrimidine phosphorothioates do (55).

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In this paper we describe the results of an investigation on the thermodynamic stability of DNA triplexes obtained from pyrimidine phosphorothioate oligonucleotides, containing an increasing number of sulphur substitutions in the phosphate backbone, directed against a R·Y duplex. Moreover, considering that triplex-forming pyrimidine and purine oligonucleotides targeted to R·Y sites located near or within a promoter sequence have been shown to inhibit transcription (25–28), we have extended this investigation by using phosphorothioate oligonucleotides as transcription repressors. We found that the 20-mer phosphorothioate oligonucleotides Y20-2POS and Y20-5POS (see Figure 7), containing 10 and 26% thioate groups respectively, strongly inhibit the transcription activity of T7 RNA polymerase *in vitro*, under different experimental conditions.

MATERIAL AND METHODS

DNA samples

The oligonucleotides reported in Figures 1 and 7 containing phosphate (PO₂) and phosphorothioate (POS) linkages have been synthesised with a DNA Synthesiser (Applied Biosystem 380 B), using a standard phosphoroamidite chemistry. Replacement of one of the non-bridging internucleotide oxygen atoms with sulphur was performed with TETD (tetraethylthiuram disulphide) in acetonitrile (56). Following the manufacturer's instructions, a sulphurization step (15 min) was introduced in the standard phosphoroamidite coupling cycle so that oligonucleotides that have both phosphodiester and phosphothioate linkages were synthesised. Deprotection of oligonucleotides was carried out with concentrated NH₃ solution at 60°C for 8 h. Crude deprotected oligonucleotides were purified by FPLC using a mono Q column with a linear gradient of ammonium bicarbonate from 0.01 to 1.0 M. We observed that sulphur-containing oligonucleotides bind to the anion exchanger more tightly than PO₂ analogues, thus requiring higher salt concentrations for elution. The purity of the oligomers was checked by 20% PAGE in denaturing conditions. The presence of POS groups in the oligonucleotides was confirmed by ³¹P NMR spectroscopy. Chemical shifts of POS groups relative to PO₂, measured at 162 MHz, fall at 56–57 ppm. The ratio of the integrated peaks PO₂/POS gave the expected values. DNA concentrations were determined by UV absorbance at 260 nm in water, using as extinction coefficients 7500, 8500, 15000 and 12500 M⁻¹ cm⁻¹ for C, T, A and G, respectively, in the denatured state (57).

Melting experiments

Melting experiments were performed on a Cary 219 spectrophotometer (Varian) equipped with a thermostated cuvette holder. The temperature was increased at a rate of 0.5°C/min with a Haake PG20 temperature programmer, connected to a Haake water circulating bath. The melting profiles were recorded at 270 nm in 0.1 M sodium acetate (pH 5), 50 mM NaCl, 10 mM MgCl₂ (standard buffer), with oligomer concentrations in the micromolar range. Experiments were also performed in 0.1 M Tris–acetate (pH 6), 50 mM NaCl, 10 mM MgCl₂.

Electrophoresis

PAGE experiments were carried out on gels (10×15×0.10 cm) obtained from buffer solutions containing 20% (w/v) acrylamide, 3.3% (w/v) bisacrylamide, 0.07% (w/v) ammonium persulfate. The electrophoreses were conducted at constant voltage of about 10 V cm⁻¹, with a current lower than 20 mA. The amount of

sample loaded was about 2 µg. The DNA bands were visualised by staining with Stains-All dye in water:formamide (1:1).

Thermodynamic analysis

The thermodynamic parameters of the triplex to hairpin plus single strand transition:

were obtained by van't Hoff analyses of UV melting curves. Two methods of analysis were followed. First, from the experimental melting curves we obtained according to an *all-or-none* model the fraction of triplex dissociation, α , as a function of temperature (Figure 5). The sigmoidal α vs T curves were then fitted to equation (b) (explicit for α):

$$K_{eq} = \frac{\alpha^2 C_T}{1-\alpha} = \left(-\frac{\Delta H}{RT} + \frac{\Delta S}{R} \right) \quad (\text{b})$$

where K_{eq} is the equilibrium constant for reaction (a). Using a non-linear best-fit program the ΔH and ΔS parameters of equation (b) were adjusted until theoretical and experimental α vs T curves coincide. Secondly, ΔH and ΔS values were determined from the concentration dependence of the melting curves:

$$\frac{1}{T_m} = -\frac{R}{\Delta H} \ln C_T + \frac{(0.22R + \Delta S)}{\Delta H} \quad (\text{c})$$

where C_T is the total concentration of h26, equal to that of Y11-S_i. A detailed description of the methods was previously reported (11–13).

Circular dichroism

Circular dichroism spectra were obtained with a Jasco J-500 A dichrograph connected to a Jasco DP 500 data processor. Spectra are presented as $\Delta\epsilon = (\Delta\epsilon_L - \Delta\epsilon_R)$, in units of M⁻¹ cm⁻¹ where the molarity is expressed in (mol of triplex) L⁻¹. The absorbance of the solvent in the short wavelength region of the spectrum (200–230 nm) was minimised using a 1 mm pathlength cuvette. All spectra have been smoothed and subtracted from the baseline obtained by filling the cuvette only with the standard buffer (at pH 5 or 6).

Enzyme digestions

Snake venom phosphodiesterase I from *Crotalus adamanteus* (SVP I) was purchased from Sigma. Reactions were performed in a total volume of 1.3 ml at 37°C. The enzyme kinetics were followed by measuring the hyperchromic effect at 270 nm associated with oligonucleotide degradation. SVP was used in 10 mM Tris–HCl (pH 8.8), by adding 1.5 µl of an enzyme solution prepared by dissolving 25 mg enzyme (0.031 U/mg) in 100 µl Tris–HCl (pH 8.8) buffer. The experimental curves were fitted to a single exponential curve from which the half times were determined.

Transcription experiments

Transcription experiments were conducted with a DNA template obtained from Bluescript KS+. In the polylinker of this plasmid we cloned, between *Bam*HI and *Hind*III, a R·Y sequence of 30 bp and, in *Hind*III, a reporter gene of 690 bp, as illustrated in Figure 7. The R·Y sequence is a selected target for pyrimidine

triplex-forming oligonucleotides: Y20, Y20-2POS and Y20-5POS. Linearization of Bluescript containing the two insertions by cleavage with *Xho*I endonuclease provided the DNA template from which a run-off transcript of 787 nucleotides was obtained. Both DNA template and triplex-forming oligonucleotides were freed of RNase by being treated with Proteinase K (Sigma), extracted with phenol/chloroform and precipitated with ethanol (58) or *n*-butanol (59). *In vitro* transcriptions were performed with T7 RNA polymerase (Epicentre Technology) in 40 mM Tris-acetate (pH 6.9), 10 mM NaCl, 6 mM MgCl₂, 2 mM spermine. The reaction mixture contained 20 U of T7 RNA polymerase, XTP (X = A, U, G, C) 10 mM, α -³²P] UTP (DuPont), DTT 10 mM, sterile H₂O to 20 μ l volume. The reaction product was analysed using a denaturing 4% polyacrylamide gel.

RESULTS

We have previously shown that the PO₂ oligonucleotides Y11, 5-methyl-cytosine analogue m5CY11 bind the Watson-Crick double-stranded stem of hairpin h26 forming triple-stranded structures stabilised by C⁺·GC and T·AT triads (11,13). The PSO oligonucleotides Y11-S_i (i = 1,2,3,4,10) have the same sequence as Y11 but an increasing sulphur substitution in the sugar-phosphate backbone (Figure 1). The potential of Y11-S_i to form triple helices with h26 was first assayed by non-denaturing PAGE at pH 5 and 6. Figure 2A shows that the mobility of h26 is retarded by all five POS oligonucleotides, indicating the formation of triple helices (11,13). It is noteworthy that the number of POS linkages introduced in Y11 influences the capacity of this sequence to bind its target. A complete transformation of h26 in triplex is only observed with oligomers Y11-S₁ and Y11-S₂, containing respectively one and two POS groups at the sequence termini. In contrast, Y11-S₃, Y11-S₄ and all-POS Y11-S₁₀, which also have POS linkages inside the sequence, are not able to produce a total conversion of h26 into triplex. At pH 6, the capacity of the POS oligonucleotides to bind h26 is significantly reduced since only Y11-S₁ and Y11-S₂ form a triple helix with h26 (Figure 2B).

In order to quantify the effect of POS linkages on triplex formation by the phosphorothioates Y11-S_i (i = 1,2,3,4,10), we

Figure 1. (A) Schematic representation of the DNA sequences: hairpin duplex (h26); pyrimidine strands Y11-S_i (i = 0,1,2,3,4,10), containing an increasing number of phosphorothioate linkages. The symbol s indicates a POS linkage. (B) Structure of internucleotide linkages: phosphodiester (PO₂) and phosphorothioate (POS) with Rp and Sp configurations about phosphorous.

performed UV melting experiments. Equimolar mixtures of h26 and Y11-S_i were melted in the standard buffer at various DNA concentrations. As observed for h26·Y11 (11) and h26·m5CY11 (13), the h26·Y11-S_i triplexes melt with a clearly biphasic profile. A typical melting profile for h26·Y11-S₁₀ is reported in Figure 3. The low temperature transition (transition 1) is due

Figure 2. (A) 20% polyacrylamide gel electrophoresis in 0.1 M sodium acetate (pH 5), 50 mM NaCl, 10 mM MgCl₂. Lanes were loaded as follows: (a) h26·Y11-S₁₀; (b) h26·Y11-S₄; (c) h26·Y11-S₃; (d) h26·Y11-S₂; (e) h26·Y11-S₁; (f) h26. Equimolar amounts of h26 and Y11-S_i were incubated for 1 h at room temperature and loaded (2 μ g DNA) in the gel thermostated at 15°C. The gel was run at 5 V cm⁻¹ and stained with 'stains all' dye. (B) 20% PAGE in 0.1 M Tris-acetate (pH 6), 50 mM NaCl, 10 mM MgCl₂. Lanes were loaded as follows: (a) h26; (b) h26·Y11-S₁; (c) h26·Y11-S₂; (d) h26·Y11-S₃; (e) h26·Y11-S₄; (f) h26·Y11-S₁₀. Sample preparation and analysis were performed as in A.

Figure 3. Thermal denaturation of the triple-stranded structure obtained from an equimolar mixture between h26 and Y11-S₁₀ in 0.1 M sodium acetate (pH 5), 50 mM NaCl, 10 mM MgCl₂. Absorbance was recorded at 270 nm in a 0.5 cm pathlength quartz cuvette containing a DNA solution at the concentration of 32.7 μ M in triplex. The low temperature transition is due to h26·Y11-S₁₀→h26 + Y11-S₁₀ (T_m = 39°C), the high temperature transition is due to h26→coil (T_m = 72°C).

Table 1. Thermodynamic parameters of triplex formation, h26·Y11-S_i (i = 1,2,3,4,10), in 0.1 M sodium acetate (pH 5), 50 mM NaCl, 10 mM MgCl₂a

Triplex	μ M	T (°C)	$-\Delta H$ (kcal/mol)	$-\Delta S$ (e.u.)	$-\Delta G^{37^\circ\text{C}}$ (kcal/mol)	%h ^b
Data from best-fit analyses						
h26 + Y11-S ₁	3.5	44	62	173	8.3	10
h26 + Y11-S ₂	3.6	42	61	170	8.3	10
h26 + Y11-S ₃	4.0	40	—	—	—	8
h26 + Y11-S ₄	5.1	38	70	205	6.4	8
h26 + Y11-S ₁₀	4.7	32	68	201	5.6	8
Data from $1/T_m = R \ln C_T/\Delta H + (\Delta S + 0.22R)/\Delta H$						
h26 + Y11				70	190	11
h26 + m ⁵ CY11				65 ^c	184 ^a	8
h26 + Y11-S ₁₀				69	201	6.6

^a T_m , ΔH and ΔS are expressed as round numbers; the uncertainty on T_m is $\pm 0.5^\circ\text{C}$, on ΔH and Δ at most $\pm 10\%$ ^b%h = $[A(55^\circ\text{C}) - A(25^\circ\text{C})]/A(25^\circ\text{C})$ ^cData from ref. 13, obtained at pH 6.

to the dissociation of the POS pyrimidine oligonucleotide from the double helix of h26, the high temperature transition (transition 2) is due to the denaturation of hairpin h26. A biphasic profile provides strong evidence that equimolar mixtures h26·Y11-S_i (i = 1,2,3,4,10) form triple helices, under the experimental conditions used. The melting data are collected in Table 1. The following observations can be made: (a) sulphurization of phosphodiester linkages destabilizes the triplex proportionally to the degree of phosphate backbone modification. The T_m s drop from 48°C to 32°C as the third strand is gradually substituted with analogues containing increasing amounts of POS linkages; (b) the hyperchromicity of transition 1 is not affected by the presence of sulphur atoms, its value is constant and amounts to $9 \pm 1\%$; (c) transition 1 is characterised by a transition width of $\delta T = 17 \pm 1^\circ\text{C}$ which remains roughly constant as a function of third strand backbone modification; (d) transition 1 is reversible at a heating and cooling rate of $0.5^\circ\text{C}/\text{min}$. When the pH of the buffer was increased from 5 to 6, only Y11-S₁ and Y11-S₂ were able to form triple helices with h26, their T_m s dropped from 44°C and 42°C to 30°C and 28°C , respectively. In contrast, Y11-S_i (i = 3,4,10) failed to form triple helices with h26, in accordance with PAGE experiments.

Since the sulphur atom has a larger van der Waals radius than that of oxygen, we performed CD experiments to see whether POS oligonucleotides induce significant distortions in the structure of a Y·R·Y triplex. We analysed all the h26·Y11-S_i triplexes of Figure 1 by CD and observed that, in the standard buffer at pH 5, they exhibited spectra which were similar to the one previously reported for unmodified h26·Y11 (11). A typical CD spectrum for h26·Y11-S₁₀ is reported in Figure 4A,B. It is noteworthy that the binding of Y11-S₁₀ to the major groove of h26 brings about a negative ellipticity at 212 nm which, according to a number of studies, can be considered as a hallmark for triple helix structures (11–12,60–62). The only difference between the CD spectra of h26·Y11 and h26·Y11-S₁₀ is due to the intensity of the positive and negative bands at 278 and 212 nm respectively: 220 and $-190 \text{ M}^{-1} \text{ cm}^{-1}$ for the PO₂ triplex, 175 and $-120 \text{ M}^{-1} \text{ cm}^{-1}$ for the POS triplex. We have observed through PAGE and UV melting that at pH 6 the sequences Y11-S_i (i=3,4,10) do not form triple helices with h26. This behaviour is supported by CD experiments. Figure 4B shows that the diagnostic band at 212 nm for h26·Y11-S₁₀ disappears as the pH is increased from 5 to 6.

To determine the thermodynamic stability of the DNA triplexes made by POS oligonucleotides, we performed van't Hoff analyses of the melting curves. From the absorbance versus temperature profiles we graphically determined the fraction α of triplex dissociation as a function of temperature for all triple-stranded structures. (Figure 5A). The sigmoidal curves obtained were fitted to an *all-or-none* model (equation 2) as described in Materials and Methods. The values determined of ΔH and ΔS are presented in Table 1. Since the triplex-to-hairpin plus Y11-S_i transition is a bimolecular process, its T_m is concentration dependent. For h26·Y11-S₁₀, a variation of 27-fold in triplex concentration changes the T_m from 29°C to 39°C . Figure 5B shows the corresponding $1/T_m$ vs $\ln C_T$ plot whose slope and y-intercept allowed us to determine the ΔH and ΔS parameters (Table 1). In order to compare thermodynamic data obtained with the same method of analysis, the variation of T_m for h26·Y11 with DNA concentration previously reported (11) was reanalyzed according to a *two-state* model (Table 1). It can be seen that in the homologous POS triplex series h26·Y11-S_i, the ΔH is roughly constant, suggesting that the triplex destabilization induced by the sulphur substitution of Y11 is not due to significant enthalpy changes.

In order to determine the extent of backbone modification necessary to make phosphorothioate oligonucleotides resistant towards snake venom phosphodiesterase I (SVP I, an enzyme which quickly degrades DNA), we examined the sensitivity of the homologous sequences Y11-S_i (i = 0,1,2,3,4,10) to SVP I by recording the hyperchromic effect at 270 nm which accompanies the enzymatic reaction. The curves obtained were fitted to a single exponential (Figure 6) and confirmed what was previously found with different nucleases (36). While the all POS sequence Y11-S₁₀ exhibited a total resistance in 30 min, the unmodified PO₂ sequence Y11 was quickly degraded with half-life of about 1.6 min. As for sequences Y11-S_i (i = 1,2,3,4), containing different amounts of POS linkages, they showed a strong resistance to the nuclease, being characterised by half-lives at least three order of magnitude higher than that of PO₂ Y11 (about 103 min).

Recent studies have shown that both pyrimidine and purine-rich oligonucleotides, each targeted to a R·Y site located near or within a promoter of a gene, are able to inhibit transcription (25–31). We have extended these findings by investigating the ability of phosphorothioate oligonucleotides containing two

Figure 4. (A) Circular dichroism spectra of triplex h26·Y11-S₁₀ (—○—); h26 (—●—); Y11-S₁₀ (—△—). Spectra were obtained with a 0.2 cm pathlength cuvette in 0.1 M acetate (pH 5), 50 mM NaCl, 10 mM MgCl₂. Both samples h26 and Y11-S₁₀ were mixed each at the concentration 13 μ M in strand. (B) Spectra for h26·Y11-S₁₀ in 0.1 M sodium acetate (pH 5), 50 mM NaCl, 10 mM MgCl₂ (—●—) and 0.1 M Tris-acetate (pH 6), 50 mM NaCl, 10 mM MgCl₂ (—△—). The diagnostic negative ellipticity at 212 nm disappears as the pH of the buffer increases from 5 to 6. Spectra are the average of four scans taken at room temperature from which were deduced the average spectrum (four scans) of the buffer alone.

Figure 5. (A) Experimental and theoretical curves for the triplex-to-hairpin plus single strand transition of equimolar mixtures between h26 and Y11-S_i (i = 0, 1, 2, 4, 10). The plot reports the fraction of triplex dissociation as a function of temperature for: h26·Y11 (—□—); h26·Y11-S₁ (—○—); h26·Y11-S₂ (—▼—); h26·Y11-S₄ (—▽—); h26·Y11-S₁₀ (—●—). The full lines are the best-fit curves obtained using equation 2 (see Materials and Methods). (B) Dependence of T_m of transition h26·Y11-S₁₀→h26 + Y11-S₁₀ on DNA triplex concentration. Data are plotted as $1/T_m$ versus $\ln C_T$, the best-fit line is also reported. From the slope and the y-intercept the parameters ΔH and ΔS have been obtained.

Figure 6. Snake venom phosphodiesterase I digestion of PO₂ and POS oligonucleotide phosphorothioates containing different amounts of POS linkages. The sequences are: Y11 (—●—); Y11-S₁ (—□—); Y11-S₂ (—△—); Y11-S₁₀ (—○—). The curves of Y11-S₃ and Y11-S₄ falling between those of Y11-S₂ and Y11-S₁ are not reported. Enzymatic reactions were carried in 1.3 ml 20 mM Tris-HCl (pH 8.8), *T* = 37°C, with a DNA concentration of 0.3 mM of base. Absorbance was recorded at 270 nm; the curves have been fitted with a single exponential function.

different levels of backbone modification, 10% in Y20-2POS and 26% in Y20-5POS, to repress the bacteriophage T7 RNA polymerase transcription *in vitro*. These 20-mer phosphorothioates are expected to form stable triple helices in a pH range wider than that observed for Y11-S₁ because their cytosine content is 45% (55% in Y11-S₁) and their ΔH (and ΔG) of triplex formation should be more favourable than that of Y11-S₁, due to their longer sequence (63). Yet, under conditions in which the third strand is in large excess with respect to the target, the stability of the resulting triplex will be further enhanced, the reaction of triplex formation being bimolecular (see plot 5B and ref. 11–13). As shown in Figure 7 the DNA template was constructed by cloning in Bluescript a R·Y target and a reporter gene. The sequence R·Y is a perfect site for triplex-forming oligonucleotides Y20, Y20-2POS and Y20-5POS (Figure 7). Transcription reactions were performed in a 20 μ l volume at 25°C, using 20 U of T7 RNA polymerase which produced a full length transcript of 787 bp. We found that at pH 6.9 T7 RNA polymerase transcription activity was greatly inhibited (> 90%) by the POS oligonucleotides at concentrations of 5 and 15 μ M. Since phosphorothioate oligonucleotides have shown to inhibit HIV reverse transcriptase by a direct interaction with the enzyme, we performed the following control experiment to prove that this did not occur also with T7 RNA polymerase (64). We performed transcription reactions in the presence of Y11, Y11-S₁, Y11-S₂, Y11-S₃ and Y11-S₄, which are nonspecific for the R·Y target and contain a percentage of thioate groups up to 40%. Figure 7B shows the results of an experiment using the nonspecific oligonucleotides at 10 μ M. Although this concentration is similar to those used for transcription experiments

Figure 7. (A) The DNA template used for transcription experiments was obtained from linearization of Bluescript KS+. The primary structures of both R·Y and triplex-forming oligonucleotides Y20, Y20-2POS and Y20-5POS are shown. The DNA template was first incubated with a triplex-forming oligonucleotide for 2 h at 25°C in 40 mM Tris-acetate (pH 6.9), 10 mM NaCl, 6 mM MgCl₂, 2 mM spermine. Then T7 RNA polymerase and triphosphate nucleotides ATP, CTP, GTP and UTP (plus [α -³²P] UTP) were added to the reaction mixture. Reactions were allowed to proceed for 1.5 h. (B) Transcriptions in the presence of a triplex-forming oligonucleotide: Y20 (lane a, 5 μ M; lane b, 15 μ M); Y20-2POS (lane c, 5 μ M; lane d, 15 μ M); Y20-5POS (lane e, 5 μ M; lane f, 15 μ M). Lane g shows a control experiment: i.e. a T7 transcription reaction in the absence of triplex-forming oligonucleotide. The reaction product was analysed on 4% PAGE. After electrophoresis the gel was dried and autoradiographed on a Kodak film. (C) Control experiment for transcription. The DNA target was transcribed in the presence of nonspecific oligonucleotides Y11 (a), Y11-S₁ (b), Y11-S₂ (c), Y11-S₃ (d) and Y11-S₄ (e), i.e. oligonucleotides containing an increasing number of thioate groups, at concentration of 10 μ M. Template transcription in the absence of oligonucleotides is shown in lane f. Note that all these nonspecific phosphate and phosphorothioate oligonucleotides do not influence transcription.

with Y20-2POS and Y20-5POS (5 and 10 μM), we did not observe significant transcription inhibition, even in the presence of the thioate-rich oligonucleotide Y11-S₄. Increasing the concentration up to 50 μM we observed a slight transcription inhibition which, however, was non-significant with respect to that observed with the triplex-forming oligonucleotides (Figure 7B). Moreover, in a separate experiment we transcribed the DNA

Figure 8. Schematic representation of the major groove of a triplex having as third strand a pyrimidine strand with all PO_2 linkages substituted with nonstereospecific (R,S) POS analogues. The POS strand is parallel bound to the purine strand of the Watson-Crick target forming Hoogsteen $\text{C}^+ \cdot \text{GC}$ and $\text{T} \cdot \text{AT}$ triads. Note that the S^{-1} atom of the POS groups with a Sp configuration are in close proximity with the $\text{O}^{-1/2}$ atom of W.C. purine PO_2 groups. This may cause electrostatic repulsion and unfavourable steric interactions leading to triplex destabilization.

template in the presence of another nonspecific 24-mer oligonucleotide d(CCCTATCGCGGTGCGGTATCCC), at $15 \mu\text{M}$, and noted that it had no effect on transcription. Finally, raising the pH to 7.9, the transcription inhibition promoted by the triplex-forming oligonucleotides was drastically reduced as a result of triplex destabilization (not shown). Thus, on the basis of these accurate control experiments we can conclude that the phosphorothioate oligonucleotides Y20-2POS and Y20-5POS inhibit T7 RNA polymerase transcription with a mechanism based on triplex formation.

DISCUSSION

In this study we have shown that phosphorothioate oligonucleotides containing different levels of phosphate backbone modifications can form stable triple helices with Watson-Crick double-stranded DNA. The substitution of a non-bridging oxygen with a sulphur atom in a phosphodiester group produces two stereoisomers (Rp, Sp) (Figure 1). Since the automated synthesis of DNA cannot be stereospecific, the POS oligonucleotides that we have synthesised are a mixture of diastereoisomers. Thus, Y11-S₁ with a single modification is a mixture of 21 isomers, whereas Y11-S₁₀ is a mixture of 210 isomers. Although PSO oligonucleotides can be prepared with a stereospecific configuration (51,54,55), their large scale production for biological testing is not convenient. In fact, antisense POS oligonucleotides are generally used as a mixture of stereoisomers (32, 65). Both PAGE and UV melting indicate that sulphur substitution in the third strand significantly influences the stability

of Y·R·Y triplexes. The data of Table 1 suggest that for every POS linkage a depression of about 2°C of the T_m of triplex-to-hairpin plus Y11-S_i transition is to be expected. A similar effect has been observed for duplexes containing in one strand all POS linkages. For 15-mer duplexes with different C+G contents a T_m depression varying from 9°C to 17°C was obtained, for phosphorothioate AT duplexes the ΔT observed was even higher (36). This effect on T_m has also been observed for polynucleotides containing one or both POS strands (49,52). Thus, experimental data support the notion that POS linkages have the effect of depressing the T_m of both duplex and triplex DNA structures. As the replacement of one oxygen with sulphur can be considered a minimal structural change, what is the physical origin of such a phenomenon? We attempted to rationalise this on the basis of the following considerations. The sulphur atom is less electronegative than the oxygen (2.5 vs 3.5, on the Pauling scale) and has a slightly larger van der Waals radius. In spite of that, the negative charge in a POS group seems to be largely localized on sulphur, because it is more polarizable than oxygen (66). This finding is supported by Raman and IR spectra, indicating that in phosphorothioate compounds the P-S bonding in a POS group is little, if at all, involved in resonance effect (66). Thus, while in PO_2 groups the negative charge is delocalized over the non-bridging oxygen, in POS groups it is essentially delocalized on sulphur. According to this view, the major groove of triplex h26·Y11-S₁₀ may be represented with a very simple model, as in Figure 8. Since the third strand in Y·R·Y triplexes lies closer to the R strand of the target duplex h26, two effects can be produced by the sulphur atoms: (a) unfavourable steric interactions between S and O, due to a larger atomic radius of the former with respect to the latter; (b) increased anion-anion repulsion between juxtaposed fully negative S^{-1} and partially negative $\text{O}^{-1/2}$, the negative charge in the former atom being not delocalized over the POS group. Thus, the higher the number of POS linkages in the third strand, the stronger will be the anion-anion repulsion and therefore the higher will be the T_m repression (52). This view is supported by the fact that the capacity of POS strands to form DNA triplexes is different for individual Rp and Sp stereoisomers. As expected, the Sp isomer, having the sulphur atoms on the side of the purine strand of the target duplex, forms a triplex with T_m 10°C lower than the corresponding Rp triplex (54).

Table 1 shows that the free energy (ΔG) of triplex formation by POS oligonucleotides becomes less favourable as the level of phosphate backbone modification increases. We find that all-thioate Y11-S₁₀ strand forms a triplex which is less stable by 5.4 kcal/mol than the triplex formed by Y11. Moreover, the enthalpy contents of triplexes h26·Y11-S_i can be considered constant, $\Delta H = 65 \pm 7 \text{ kcal/mol}$, within experimental error ($\pm 10\%$ for van't Hoff analysis). This result is corroborated by circular dichroism spectroscopy. In fact, the CD spectra of normal and phosphorothioate triplexes are similar, suggesting that the POS strands Y11-S_i do not seem to induce significant structural changes on the triple-stranded structures. Hence, the destabilization of the triplex induced by POS oligonucleotides might be entropic in origin. This is supported by the finding that the enthalpy contents measured for PO_2 duplexes with several (C+G) amounts is the same as that found for the POS duplex analogues, although the former are significantly more stable than the latter (36). However, more experiments have to be performed to get better insight into the effect of sulphur atoms on the ΔG of triple-stranded DNA.

Recently it has been reported that the all-thioate 17-mer oligonucleotide d(TTTCTTTTCTTTT), either in the Rp configuration or as a (Rp,Sp) diastereomeric mixture, did not significantly bind to its target (55). This is in contrast with the finding that all-thioate Y11-S₁₀ binds to h26. However, it should be noted that these oligonucleotides have rather different sequences, because Y11-S₁₀ contains 54% cytosines, while the 17-mer contains only 12%. Probably their different capacity of forming a triplex may be due to sequence effects.

The nuclease susceptibility of POS oligonucleotides has been investigated by several authors (36–37,49). In general POS oligonucleotides are found to be more resistant than PO₂ analogues towards both endo- and exonucleases. This difference is pronounced with SVP I. We found that POS oligonucleotides are at least three orders of magnitude more resistant to SVP I than PO₂ analogues. Moreover, considering that: (i) 3' and 5' capped oligonucleotide phosphorothioate Y11-S₂ retains the nuclease resistance of all-POS Y11-S₁₀; (ii) a similar behaviour has been observed by other capped POS oligonucleotides towards nuclease S1 and P1 (36); (iii) the capacity of POS oligonucleotides to form triple helices is inversely proportional to the number of POS linkages, the oligonucleotides with capped POS ends may be considered as good choices for use as *gene blockers* in antisense strategy.

In vitro experiments have shown that the POS oligonucleotides Y20-2POS and Y20-5POS are able to inhibit transcription of T7 RNA polymerase via triple helix formation at pH 6.9. Since the 20-mer oligonucleotides Y20-2POS and Y20-5POS contain a low level of phosphate modification, 10% and 26% respectively, and were used in large amounts with respect to the template (mole oligo/mole template = 125 and 375), the POS oligonucleotides were able to form stable triple helices even at a value of pH near to neutrality. Transcription repression from prokaryotic promoters by triplex-forming oligonucleotides has been previously reported (25–28,67). However, in these studies the R·Y target site was either adjacent or partly overlapping the RNA polymerase promoter. In contrast, we constructed a DNA template bearing the R·Y site 46 bp (about 4 helical turns) downstream from the T7 promoter. Since we also observed a strong transcription inhibition, we can conclude that the target for triple helix formation cannot necessarily be confined to the promoter region (67,68). A systematic investigation on the transcription repression by unmodified and modified oligonucleotides observed under different experimental conditions will be published elsewhere.

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