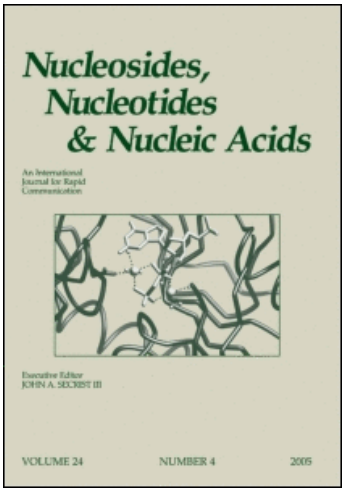

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OLIGONUCLEOTIDE CONJUGATES DESIGNED FOR DISCRIMINATIVE HYBRIDIZATION AT PHYSIOLOGICAL TEMPERATURE.

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ABSTRACT: We report the synthesis of oligonucleotide conjugates engineered to allow discriminative hybridization at temperatures around physiological. Two types of structural modifications were introduced: 1) internal oligomethylene and oligoethylene glycol spacers, and 2) terminal phenazinium residues. The thermal denaturation behaviour of the complexes formed by these oligonucleotide conjugates with a target sequence is compared to that of natural duplexes. We observed a lowering of the T_m of the duplexes formed by the internal modified oligonucleotides, whilst the terminal phenazinium residues enhance their stability. The effect of the spacers is modulated by their length and hydrophobic or hydrophilic nature. Alkylating substituents, which modify the target DNA strand on hybridization, were introduced on all conjugates, and the target cleavage obtained after piperidine treatment used as a further indicator of hybridization.

INTRODUCTION

The use of oligonucleotides, their analogues and derivatives for the control of expression of potentially pathogenic gene products is an intriguing therapeutic concept, since it will allow drug design on a rational basis. In order to be applicable as therapeutics, oligonucleotides have to be structurally modified, so that they will penetrate into cells and be stable to degradation within a biological environment. Since the control of gene expression depends primarily on Watson-Crick base pairing, a major requirement is for stable and discriminating hybridization. Most work done on this topic has concentrated on achieving utmost stability of complexes of antisense oligonucleotides with the target mRNA, which means that mismatch discrimination will require

hybridization at temperatures well above 37 °C, which may limit their applicability in vivo. In recent work[1] we have engineered antisense oligonucleotides to allow stringent hybridization at temperatures around physiological. This was done by introducing two types of structural modifications: 1) internal oligomethylene and oligoethyleneglycol spacers, which destabilize the double-helical arrangement of oligonucleotide to target; 2) terminal phenazinium residues, which enhance the stability of hybridization.

Another focus of our studies has been the introduction of alkylating substituents, which modify the target DNA strand on hybridization[2]. The strand cleavage obtained after piperidine treatment was subsequently used as an indicator of hybridization.

In this paper we report the combined effects of the aforementioned modifications on a sample antisense oligonucleotide aimed to optimize its hybridization properties at physiological temperature.

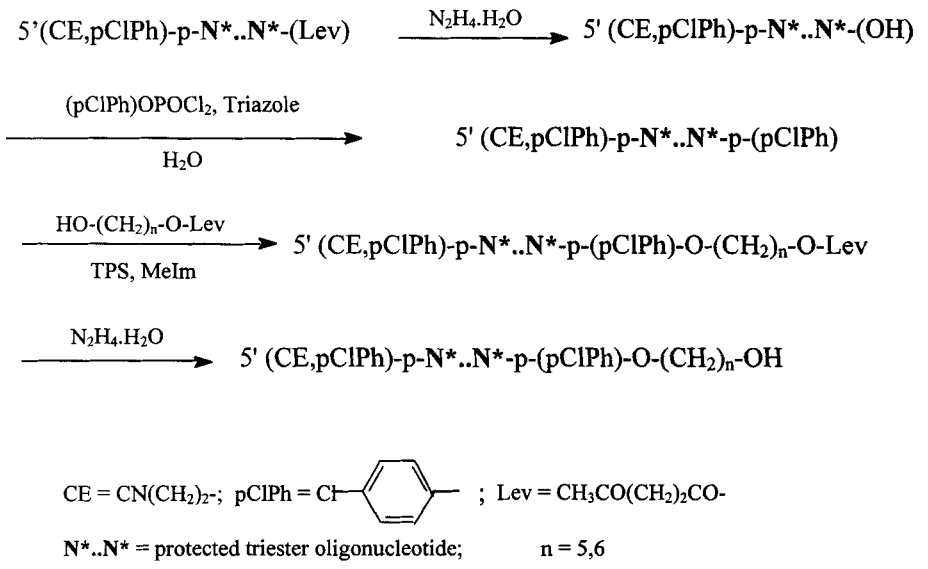
RESULTS

Synthesis of oligonucleotides with non nucleotidic spacers and their derivatives substituted with phenazinium and alkylating residues.

For these studies a fragment of HIV-1 negative RNA, 20 bases long, was chosen as DNA- template[3,4]. This oligonucleotide was synthesized, together with a number of complementary oligonucleotides and their analogues.

Native and "chimeric" antisense oligonucleotides with inserted pentane-1,5-diol- or hexane-1,6-diol-moieties (Scheme 1) were prepared by using phosphotriester solution synthesis starting with [5'-(p-chlorophenyl)(β-cyanoethyl)]-N-acyl-(3'-O-levulinyl)-nucleoside-5'-phosphates as synthons[5]. The overall yields were 80-90%. This approach permits to prepare the completely protected oligonucleotides bearing, simultaneously, 5'- and/or 3'-phosphates, which facilitates the attachment of additional chemical groups to the termini. After the final deblocking with TBAF[6], oligonucleotides and chimeras were purified by ion-exchange and reverse phase HPLC. The increased hydrophobicity conveyed by introduction of the oligomethylene spacers is reflected by the increased retention time in reversed phase chromatography.

„Chimeric" oligonucleotides of the same sequence containing hydrophilic hexaethylene glycol spacers were prepared on a Pharmacia Gene Assembler mechanized synthesizer using phosphoramidite synthons[7]. The yields of cycles involving the hexaethylene glycol synthons were at least 80%.



SCHEME 1: Solution synthesis of chimeric oligonucleotides: example of the introduction of an oligomethylene spacer at the 3' end of the oligonucleotide.

Polyaromatic dye molecules, e.g. 2-(N-hydroxyethyl-)phenazinium (Phn) residues, coupled to the 5'- or 3'-terminal phosphate residue of oligonucleotides, are known to increase their strength of hybridization to complementary sequences[8]. Therefore, the phenazinium residue was attached to a chimeric octanucleotide as an option to stabilize its complementary complexes. The attachment of the phenazinium residue to the 3'-end of octanucleotides was done in accordance with the approach developed earlier by us[8].

The 5'-terminal addition of alkylating residues was carried out by reaction of the activated 5'-phosphate residue with 4-(N-methylaminomethyl-)-N-methyl,N-chloroethylaniline (RCl)[9].

Hybridization properties of chimeric oligonucleotides.

The influence of the insertion of aliphatic spacers on the hybridization properties of oligonucleotides was studied by looking at duplex formation of HIV-1-specific eicosanucleotides with respective "chimeric" complementary oligonucleotides of various length. As references we used duplexes formed with the same non-spacered sequences. Additionally, the formation of gapped duplexes was studied, where the two non

interlinked fragments served as hybridization partners. The arrangement of the different hybridization partners and their melting temperatures are represented in the Table.

Attempts at the formation of gapped duplexes between the template and pairs of neighboring tetranucleotides (**1**) were not successful; obviously these oligonucleotides are too short for stable hybridization.

The "chimeric" oligonucleotides, in which the two tetranucleotide segments are connected by a hexamethylene-oxy-spacer, is capable of duplex formation, but the stability is marginal with T_m <7°C (**2**). If, on the other hand, the same sequences are used with a terminal phenazinium substituent, the hybrids are stabilized with T_m = 13°C (**3**). The same effect was observed with the full length octanucleotide (**4**): the T_m increases from 35°C to 43°C with the introduction of the phenazinium substituent (**5**).

By increasing the length of both natural and "chimeric" oligonucleotides, an enhanced stability of the corresponding hybrids is observed. The gapped duplex between the template and the neighboring 8mer and 4mer (**6**) shows a maximum in the differential melting curve at 32°C. As expected, the "chimeric" oligonucleotides, in which the oligonucleotide moieties are joined by a spacer (**7a, 7b**), indicate a higher duplex stability, while an even more stable duplex is formed by the natural dodecanucleotide (**8**). Noteworthy, the comparison between the hybridization properties of the "chimeric" 12mers carrying different alkanyldiol segments (pentane-1,5-diol and hexane-1,6-diol) allows to observe the influence of the spacer length. In fact, the spacer one single methylene group longer (**7b**) decreases the T_m of the complex by 3°C. Moreover, the replacement of the hexane-1,6-diol spacer by the longer hexaethylene glycol unit causes an even more dramatic reduction of the stability of the complexes, as demonstrated with chimeric 20-mer oligonucleotides, where the observed T_m for the native duplex (**9**) is 69°C. The oligonucleotide in which the chain is interrupted by two hexane-1,6-diol-spacers shows a T_m of the duplex (**10**) of 51°C (ΔT_m=18°C), but if two hexaethyleneglycol spacers are introduced, the T_m of the duplex (**11**) is lowered to 45 °C (ΔT_m=24°C).

DNA-template modification by alkylating agents bound to chimeric oligonucleotides

The introduction of a reactive residue, 4-(N-2-chloroethyl-N-methylamino)-benzyl-methyl amine, as an alkylating agent bound to the end of the oligonucleotide chain

TABLE 1: Duplexes formed by the template and natural and chimeric oligonucleotides and their melting temperatures. Spacers used: "**V**" = -p-O-$(CH_2)_5$-O-p-; "**Y**" = -p-O-$(CH_2)_6$-O-p-; "**W**" = -p-O-$(CH_2$-CH_2-$O)_6$-O-p-.*T_m of this complex does not differ from the T_m of the template and the native octanucleotide alone. The thermal denaturation of oligonucleotide complexes was performed in 0.01M sodium cacodylate buffer (pH 7.4) with 0.1M NaCl, 1mM EDTA, the concentration of each oligonucleotide component was 13μM[8].

Complex Nr.	5'pTGCCTGGAGCTGCTTGATGC (template) +	T_m °C
1	ACCTp CGACp5'	not detectable
2	ACCT-**Y**-CGACp5'	<7
3	Phn-pACCT-**Y**-CGACp5'	13
4	ACCTCGACp5'	35
5	Phn-pACCTCGACp5'	43
6	ACGGACCTp CGACp5'	32*
7a	ACGGACCT-**V**-CGACp5'	41
7b	ACGGACCT-**Y**-CGACp5'	38
8	ACGGACCTCGACp5'	55
9	ACGGACCTCGACGAACTACGp5'	69
10	ACGGACCT-**Y**-CGAC-**Y**-GAACTACGp5'	51
11	ACGGACCT-**W**-CGAC-**W**-GAACTACGp5'	45

allows to investigate the duplex formation without influencing its hybridization properties.

A 5'-[32P]-DNA template was treated with several alkylating oligonucleotides at 20 °C for 48 h, followed by a piperidine treatment, which cleaves at alkaline-labile positions. The alkaline-stable products of template modification at the C^{13} position (adducts with lower mobility in the Figure) were separately identified by preliminary treatment with hydrazine hydrate (data not shown)[12].

The effect of chemical modification generated by the "chimeric" compounds was compared with that produced by the native octanucleotide, which forms a stable complex with the template: this native octanucleotide modifies the template effectively (lane c. Extent of modification: 77%). The relative chimeric octanucleotide gave no DNA modification (lane d), very likely due to the low T_m value observed for the complex. When a stabilizing phenazinium residue is introduced at the 3'-end, the extent of modification is increased (data not shown). Alkylating derivatives of longer oligonucleotides, which form more stable duplexes, give higher modification of the

template: the extent of DNA modification achieved with alkylating reagents bound both to native (lane a) and chimeric (lane b) dodecanucleotides was 60% and 64% respectively. In all cases the reagents span their activity through a wide range of bases (G7, G9, G12, C13, G16 and G19), with a site of maximum alkylation at the C13 position (Figure).

EXPERIMENTAL SECTION

Synthesis of oligonucleotides

Oligonucleotides were synthesized by the phosphotriester method starting from 5'-*p*-chlorophenyl-*N*-acyl-3'-*O*-levulinyl-nucleoside-5'-phosphates and 5'(*p*-chlorophenyl)-(*β*-cyanoethyl)-*N*-acyl-nucleoside-5'-phosphates[5]. The insertion of pentane-1,5-diol- or hexane-1,6-diol- moieties into "chimeric" oligonucleotides was done using the same strategy (Scheme 1). 3'-N(2-Hydroxyethyl)phenazinium derivatives of oligonucleotides were obtained according to reference[8]. Alkylating derivatives of oligonucleotides containing 4(N-2chloroethyl,N-methylamine)benzylmethylamide were synthesized using protocol[9] and analyzed by HPLC on a LiChrosorb RP-18 column using a gradient of acetonitrile in 0.05 M $LiClO_4$. The oligonucleotide containing hexaethyleneglycol segments was synthesized on a Pharmacia automated synthesizer using phosphoramidites chemistry. Hexaethyleneglycol was purchased from Aldrich and converted to α-(dimethoxytrityl)-hexaethyleneglycol-ω-(β-cyanoethoxy)(N,N-diisopropyl)phosphoramidite[7]. After assembly, the "trityl-off" oligomere was treated with concentrated ammonia (10h at 50°C) and purified on a Merck LiChroCart 125-4 LiChrospher 100 RP18 (5 micron) column using a gradient of acetonitril from 10% to 30% over 20 minutes, in 0.1M triethylammonium acetate buffer pH7 at 0.8ml/min.

Physical measurements

The concentrations of oligonucleotides and their derivatives were measured spectrophotometrically using extinction coefficients ε_{260} of mono- and dinucleotides[13], of the alkylating group ($1.47 \times 10^4 M^{-1}cm^{-1}$)[9], and of the N-(2-hydroxyethyl)phenazinium residue ($1 \times 10^4 M^{-1}cm^{-1}$)[8].

The thermal denaturation of oligonucleotide complexes was performed in 10 mM sodium cacodylate (pH 7.4), containing 0.1 M NaCl, 1 mM EDTA, the concentration of each component of complex was 1.3×10^{-5}M. The absorption at 260 nm was detected for each

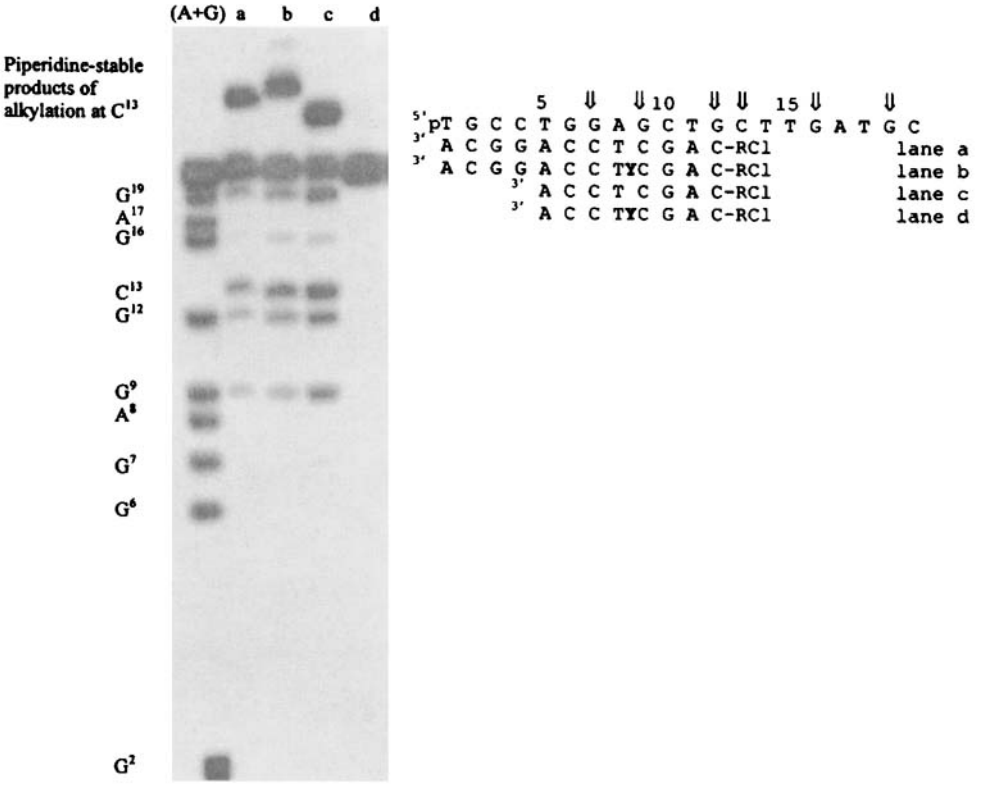


FIG 1: Autoradiogram of the 20% denaturating polyacrylamide gel-electrophoresis of DNA template modification, after piperidine treatment. Lane (A+G): products of the (A+G) sequencing reaction[10].
Conditions as reported in the experimental section.
Y = -p-O-$(CH_2)_6$-O-p-
RCl = 4-(N-2-chloroethyl-N-methylamino) benzylmethylamine

oligonucleotide mixture as a function of increasing temperature, heating rate was 0.5-1°C/min on the UV detector of Millichrom liquid chromatograph in a thermoregulated cell (pathlength 1.3mm) specially designed for this purpose. The equilibrium optical melting curves were found on the basis of more than 600 experimental points with the step 10 point/°C and were completely reversible in heating-cooling processes. The first derivatives of optical melting curves versus temperature were calculated using a gradient of linear approximation by 10 experimental points.

Modification of DNA fragments with alkylating oligonucleotide derivatives

5'-[32P]-Labeled oligonucleotide template was obtained according to protocol[14]. Modification of this template was carried out in 0.1 M NaCl, 0.01 M tris-HCl (pH 7.2), 1 mM EDTA at 20°C for 48h (> 5 half-times of the reaction of ionization of C-Cl-bond in the reagents)[11] . The concentration of target was 5 x 10^{-7}M, the concentration of the oligonucleotide reagent 1 x 10^{-5}M. The cleavage of the modified DNA fragment after alkylation was accomplished by a treatment with 1 M piperidine at 100°C for 30-50 min[12]. The cleavage products were resolved by electrophoresis on a 20% polyacrylamide 7M urea gel. After autoradiography the radioactive spots were cut off the gel and counted by liquid scintillation on a Mark III ("Nuclear Chicago",USA) counter. The modification extents were calculated as the ratio of radioactivity of the spot of product to the sum of radioactivities of the spots of product and unchanged DNA target.

CONCLUSIONS

From all these experiments, it is possible to recognize a modulating effect on the duplex formation produced by the introduction of non-nucleotidic spacers and stabilizing terminal residues.

In particular, it appears possible to lower the T_m of the complexes to a near-physiological temperature level and to maintain the recognizing capability of the sequences.

In this way, by a careful combination of intercalating, stabilizing moieties, together with a balanced choice of number and quality of non-nucleotidic spacers, it seems possible to tailor oligonucleotide sequences that offer a maximum discrimination of the target and a higher power of interaction at physiological temperature.

Further, detailed studies of the RNase activation properties and of the biological activities of the synthesized "chimeric" oligonucleotides, that will undoubtedly give a better insight on the efficiency of these compounds, are in progress.

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