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Antisense properties of tricyclo-DNA

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ABSTRACT

Tricyclo (tc)-DNA belongs to the class of conformationally constrained DNA analogs that show enhanced binding properties to DNA and RNA. We prepared tc-oligonucleotides up to 17 nt in length, and evaluated their binding efficiency and selectivity towards complementary RNA, their biological stability in serum, their RNase H inducing potential and their antisense activity in a cellular assay. Relative to RNA or 2′-*O*-Me-phosphorothioate (PS)-RNA, fully modified tc-oligodeoxynucleotides, 10–17 nt in length, show enhanced selectivity and enhanced thermal stability by ~1°C/modification in binding to RNA targets. Tricyclodeoxyoligonucleotides are completely stable in heat-deactivated fetal calf serum at 37°C. Moreover, tc-DNA–RNA duplexes are not substrates for RNase H. To test for antisense effects *in vivo*, we used HeLa cell lines stably expressing the human β-globin gene with two different point mutations in the second intron. These mutations lead to the inclusion of an aberrant exon in β-globin mRNA. Lipofectamine-mediated delivery of a 17mer tc-oligodeoxynucleotide complementary to the 3′-cryptic splice site results in correction of aberrant splicing already at nanomolar concentrations with up to 100-fold enhanced efficiency relative to a 2′-*O*-Me-PS-RNA oligonucleotide of the same length and sequence. In contrast to 2′-*O*-Me-PS-RNA, tc-DNA shows antisense activity even in the absence of lipofectamine, albeit only at much higher oligonucleotide concentrations.

INTRODUCTION

Genome sequencing has identified many genes that encode proteins whose functions are yet unknown. A number of these proteins will undoubtedly be future targets in medicinal chemistry. Understanding their cellular roles requires inhibition of their gene expression. Besides this there is a growing therapeutic interest in the inhibition of disease-related proteins on the genetic level. Oligonucleotides are potent synthetic tools for such applications, because knowledge of the target gene sequence provides sufficient information for inhibitor design (antisense oligonucleotides) (1,2). However, the relatively weak affinity to complementary RNA and the short cellular half-life of unmodified oligodeoxynucleotides requires the use of analogs.

The first generation of analogs that are now widely used in antisense applications are the phosphorothioate (PS) oligodeoxynucleotides, in which one non-bridging oxygen in the phosphodiester unit is replaced by sulfur. PS-oligonucleotides show increased resistance to enzymic degradation while maintaining the properties of unmodified deoxyoligonucleotides to elicit RNase H activity (3). If coding regions of a given gene are targeted, RNase H-mediated degradation of the mRNA at the complexation site is an important antisense mechanism (4,5). The relatively weak binding to RNA, however, requires high PS-oligonucleotide concentration, which is often accompanied by undesired and toxic non-antisense effects *in vivo* (6).

Second generation antisense oligonucleotides include the 2′-*O*-alkyl-RNAs with the 2′-*O*-methyl- and the 2′-*O*-methoxyethyl -RNA being the most prominent representatives (7). Typically, these analogs show enhanced biostability and enhanced RNA affinity compared with DNA. Due to the fact that pure 2′-*O*-alkyl-oligonucleotides do not elicit RNase H activity, strategies involving chimeric oligonucleotides with a window of deoxyribonucleotide units in the center of the sequence, allowing for RNase H activity (gapmers), were developed (8). With respect to therapeutic applications, about 12 antisense oligonucleotides of the first and second generation are now in clinical trials. One PS-oligodeoxynucleotide (Vitravene™) for the treatment of cytomegalovirus-induced retinitis was recently approved for commercialization.

There is an ongoing challenge in the discovery of alternative DNA or RNA structures with improved chemical and biological properties. Besides advances in the field of the charge-neutral polyamide nucleic acid analogs (PNAs) (9–11), the concept of conformational pre-organization has recently proven to be very successful and has led to a number of oligonucleotide analogs with improved RNA-binding properties and enhanced biostability (12–14). Particularly noteworthy are the DNA-analog hexitol-NA (HNA) (15,16) and the RNA analog locked nucleic acid (LNA) (17,18). Both analogs show enhanced RNA binding relative to the 2′-*O*-alkyl RNAs and interesting antisense properties. A recent contribution from our laboratory is the analog tricyclo (tc)-DNA (Fig. 1) (19–21). tc-oligodeoxynucleotides can be synthesized using standard phosphoramidite chemistry, are resistant to 3′-exonucleases and show strong binding properties to complementary RNA. The high thermal stability of tc-DNA–RNA

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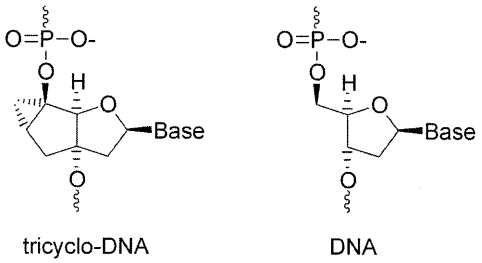

Figure 1. Representation of the chemical structure of tc-DNA as compared with DNA.

duplexes prompted us to further investigate its biological properties and its potential for antisense applications.

The results from the present study suggest that tc-oligodeoxynucleotides up to 17 nt in length still recognize complementary RNA with higher affinity compared with 2′-*O*-Me-PS-RNA. Furthermore, tc-oligodeoxynucleotides are stable in serum, do not activate RNase H and show up to 100-fold increase in antisense activity relative to 2′-*O*-Me-PS-RNA in a cellular model measuring the correction of aberrant splicing of human thalassemic β-globin mRNAs.

MATERIALS AND METHODS

Synthesis of oligonucleotides

tc-oligodeoxynucleotides were prepared on the 1.3 µmol scale on a Pharmacia Gene Assembler Special™ DNA-synthesizer using an adapted phosphoramidite chemistry procedure described in detail previously (20). Briefly, the decamers tcd(pAACTGTCACG) and tcd(pCGTGACAGTT) were assembled on a universal solid support (CT-Gen, San Jose, CA) and equipped with a 5′-terminal phosphate group (chemical phosphorylation reagent; Glen Research, Sterling, VA) to ensure chemical stability. The antisense octadecanucleotide (AS) tcd(pCATTATTGCCCTGAAAGa) and the control oligonucleotide (NS) tcd(pTACCCTTACCTCTCAgTc) were prepared in analogy, starting from solid supported natural deoxynucleosides, giving rise to a natural nucleotide residue (lowercase letters) at their 3′ end. The coupling time was set to 6 min and the usual activator tetrazole was replaced by the more active 5-(*S*-benzylthio)-1*H*-tetrazole (0.25 M in CH_3CN) or 5-(ethylthio)-1*H*-tetrazole (0.25 M in CH_3CN). Coupling yields were in the range of >95% per step. Post-synthetic deprotection and cleavage from solid support was performed in a standard manner (concentration NH_3, 16–64 h, 55–65°C). Crude oligonucleotides were purified by DEAE ion exchange HPLC and their purity controlled by reversed phase HPLC. The nucleotide composition of the four tcd-oligonucleotides was confirmed by ESI-TOF-MS. Oligodeoxyribonucleotides and oligoribonucleotides were prepared according to standard methods. The 2′-*O*-methyl oligoribophosphorothioate, 2′-*O*-Me-PS-r(CATTATTGCCCTGAAAGA), was from Eurogentec.

RNase H experiment

Standard manipulations of oligonucleotides and standard laboratory procedures were performed as described previously (22). Oligonucleotide tcd(pCGTGACAGTT), or its DNA and RNA equivalent (0.2 µM, in 60 mM Tris–HCl pH 7.5, 2 mM DTT, 60 mM KCl and 10 mM $MgCl_2$) were mixed with their RNA complement r(AACUGUCACG) (0.1 µM, same buffer) that was radiolabeled at its 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase, to a total volume of 30 µl. The mixture was heated to 80°C, annealed at 37°C and incubated with 0.6 U *Escherichia coli* RNase H (Roche Diagnostics). Aliquots of 7 µl were taken at regular time intervals. The reaction was quenched with gel loading buffer and the mixture loaded on a 20% denaturing polyacrylamide gel. Visualization was performed on a phosphorimager.

Serum stability tests

Thirty micrograms of the decamer tcd(pCGTGACAGTT) and its DNA equivalent were dissolved in 400 µl of Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) each, and incubated at 37°C with 45 µl of 10% heat-deactivated (65°C, 30 min) fetal calf serum (FCS; Gibco BRL). Aliquots of 100 µl were drawn after regular time intervals and mixed with 100 µl of 9 M urea in 2x TBE buffer. Of these solutions, 100 µl was mixed with 50 µl 3 M NaOAc and ethanol precipitated (250 µl EtOH, –70°C for 4 h then –20°C for 12 h). After centrifugation for 30 min at 0°C the supernatant was discarded, the pellet dissolved in 30 µl H_2O, lyophilized, and the residue taken up in TBE buffer (10 µl) and applied on a 20% denaturing polyacrylamide gel. The antisense oligonucleotide tcd(pCATTATTGCCCTGAAAGa) was treated in the same way. Aliquots were drawn after 0, 30, 180 and 360 min. Reactions were quenched with 9 M urea in 2× TBE buffer and phenol extracted. The aqueous phase was ethanol precipitated and the pellet washed with 70% EtOH. The pellet was dissolved in 10 ml TBE buffer and applied on 20% denaturing polyacrylamide gel. Visualization of oligonucleotides was performed by staining with 'stains all' (40 mg 'stains all' in 400 ml formamide:H_2O 1:1).

UV-melting curves

These were recorded on a Varian Cary-100 UV/VIS photospectrometer. A heating–cooling–heating cycle in the temperature range of 0–90°C with a gradient of 0.5°C/min was applied. Melting and annealing curves were superimposable in all cases, indicating equilibrium binding under the conditions applied. T_m values were defined as the maximum of the first derivative of the melting curves.

Cell culture, transfections and RNA analysis

HeLa cells stably expressing thalassemic human β-globin genes carrying the mutations IVS2-654 or IVS2-705 (23,24) were cultured and transfected with varying amounts of oligonucleotides, complexed with 6 µl (12 µg) lipofectamine (Invitrogen/Life Technologies), as described previously (25). For transfection without lipofectamine, 200 µl of DMEM thoroughly mixed with varying amounts of oligonucleotides were incubated with the cells in a 24-well plate for 1 h at 37°C, then 800 µl of DMEM was added for further incubation. Total cellular RNA was isolated 48 h after the start of transfection and β-globin mRNA was analyzed by reverse transcription-polymerase chain reaction (RT–PCR) as described previously (25).

RESULTS

For the following experiments, we synthesized and characterized the tc-oligonucleotides depicted in Table 1. These oligonucleotides, together with that carrying the DNA (d) or the RNA (r)

Table 1. Sequence information and ESI$^-$-TOF mass spectrometric characterization of the tc-oligodeoxynucleotides used in the present study

	Sequence	$[M-H]^-$ (calculated)	$[M-H]^-$ (found)
tc1	(pCGTGACAGTT)	3503.2	3503.1
tc2	(pAACTGTCACG)	3472.1	3472.2
tcAS	(pCATTATTGCCCTGAAAGa)	6209.2	6208.3
tcNS	(pTACCCTTACCTCTCAgTc)	6034.0	6036.0

$[M-H]^-$ is the molecular anion.

backbone as a reference, were used in order to determine the biological stability and the antisense properties of tc-DNA.

Stability and mismatch discrimination in duplexation of complementary RNA

First, we investigated the thermal stability of duplexes with complementary RNA in the given sequence context by means of UV-melting curve analysis. For the tc-DNA decamer tc2 an increase in duplex stability by +1.9°C/modification (mod.) relative to DNA (d2), and by +0.9°C/mod. relative to RNA (r2), was observed (Table 2). This clearly demonstrates the enhanced RNA affinity of tc-oligodeoxynucleotides relative to RNA itself.

An assessment of the impact of base mismatches on duplex stability was performed in the same sequence context with complementary RNA decamers displaying mismatched bases in the center of the sequence (tcT/rX). Relative to the matched tcT/rA base pair, a typical mismatch leads to an averaged decrease in T_m of 16°C in the tc-DNA–RNA duplex, as can be seen from the ΔT_m data compiled in Table 2. As expected, the T/rG wobble base pairs are nearly isoenergetic to the T/rA base pairs in both the tc-DNA–RNA and the DNA–RNA hybrids. Interestingly, the tc-DNA backbone shows the highest discriminating power, followed by the DNA and the RNA backbone. Relative to DNA, the gain in selectivity is in the range of 1.1–2.5°C/mismatch, and relative to RNA in the range of 2.6–4.8°C/mismatch.

We also measured the duplex stability with complementary RNA in the context of the AS sequence that was later used for *in vivo* experiments. We found a T_m of 77.6°C for the tcAS–RNA duplex (Fig. 2). This compares with a T_m of 62.3°C for the 2′-*O*-Me-phosphorothioate heptadecaribonucleotide (2′-*O*-Me-PS-rAS) CAUUAUUGCCCUGAAAG that was used as the reference, with the same target RNA. Thus, tc-DNA shows an averaged increase in T_m by about +0.9°C/mod. relative to 2′-*O*-Me-RNA also in a longer sequence context. However, we note that, in this comparison, we have neglected the contribution to binding of the 3′-terminal deoxyadenosine unit in tcd-AS.

Serum stability

To determine the enzymatic stability of tc-DNA in a biological environment, we incubated the oligonucleotide decamer tc1 in heat-deactivated FCS. As can be seen from Figure 3A, there is no degradation of tc1 over a time span of 5 h. Within the same time span, most of the DNA control sequence d1 was degraded. The same experiment with the tcAS oligonucleotide led to the rapid formation of a new band with a slightly higher mobility relative to the band of the original oligonucleotide. This new band was attributed to the oligonucleotide with the 3′-terminal natural deoxyribonucleoside unit lost (Fig. 3B). No further degradation was observed over the time span of 6 h, underlining the serum stability of the tc-DNA core sequence.

RNase H activation

RNase H activity leads to the degradation of the RNA strand in DNA–RNA duplexes and constitutes an important antisense mechanism. We tested the RNase H susceptibility of tc-DNA–RNA duplexes in the case of the decamer tc1. As can be seen from Figure 4, no signs of RNA degradation can be found after incubation with *E.coli* RNase H after 5 h in the duplex with tc1. Under the same conditions, cleavage of the RNA strand in the duplex with d1 is fast and shows a preference for cleavage at nucleotides C7 and G5 in the center of the sequence (positive control). As expected, the pure RNA duplex remains intact (negative control). Thus, the clear picture emerges that tc-DNA is unable to elicit RNase H activity.

Correction of aberrant splicing of thalassemic β-globin mRNA

To test for antisense effects of tc-DNA *in vivo*, we used a tissue culture model for alternative splicing established by Kole and co-workers (23,24). Two β-thalassemic mutations generate new 5′-splice sites (ss) at positions 654 and 705 in the second intron of the human β-globin gene. Because these mutations activate a common cryptic 3′-ss at position 579/580 of the same intron, an aberrant exon containing an in-frame stop codon is included in the mRNA and β-globin production is abolished. Antisense 2′-*O*-Me-phosphorothioate oligoribonucleotides targeted to the aberrant 5′- and cryptic 3′-ss can block the aberrant and restore the correct splicing pattern by forcing the splicing machinery to reselect the existing correct ss in cultured HeLa cells stably expressing the mutant β-globin genes (23,24).

To be able to study antisense effects in both mutant backgrounds, we used sequences complementary to the cryptic 3′-ss,

Table 2. T_m data from UV-melting curves at 260 nm in 10 mM NaH_2PO_4, 150 mM NaCl at pH 7.0

(pAACTGT**C**ACG)	r(pCGUG**X**CAGUU)						
	X = A	X = U		X = G		X = C	
	T_m	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m
r2	52.2°C	39.5°C	–12.7°C	50.6°C	–1.6°C	39.2°C	–13.0°C
d2	42.5°C	27.5°C	–15.0°C	40.4°C	–2.1°C	28.0°C	–14.5°C
tc2	61.6°C	44.1°C	–17.5°C	59.0°C	–2.6°C	46.0°C	–15.6°C

Duplex concentration = 2 μM.

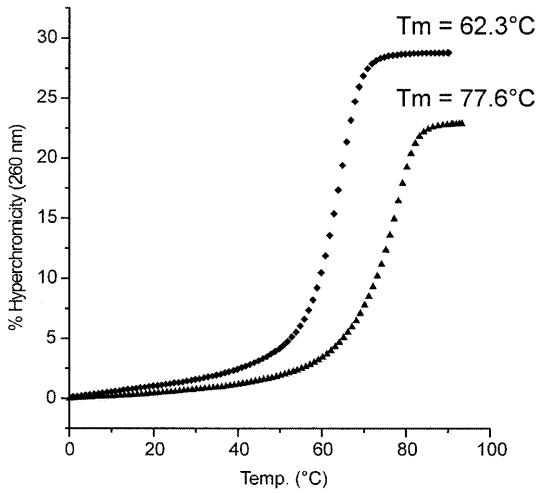

Figure 2. UV-melting curves (260 nm) of the duplexes of 2′-*O*-Me-PS-AS (diamonds) and tcd-AS (triangles) with the corresponding RNA complement r(pUCUUUCAGGGCAAUAAUG) in 10 mM NaH_2PO_4, 150 mM NaCl, pH 7.0 (2 μM duplex concentration).

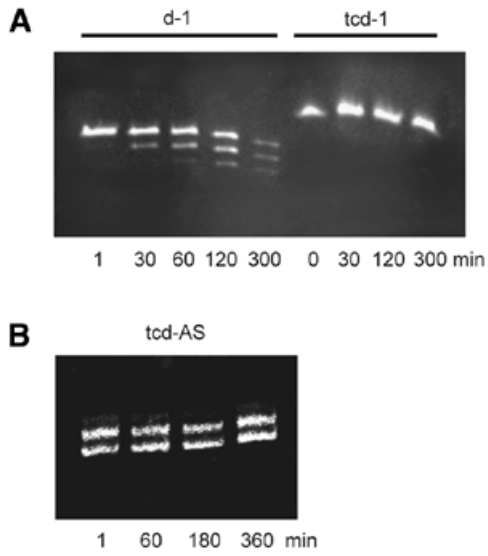

Figure 3. Polyacrylamide gel (20%) showing the time course of the FCS mediated degradation of oligonucleotides d-1 and tcd-1 (**A**) and tcd-AS (**B**) at the indicated time intervals. Bands were visualized by staining with 'stains all'.

CUUUCAG'GGCAAUAAUG (ss indicated by apostrophe). As shown previously (24), the 17mer 2′-*O*-Me-PS-rAS (identical to ON-3′CR of that paper) corrected aberrant β-globin mRNA splicing only very weakly in the IVS2-654 background (Fig. 5A, lanes 10–15), but more strongly, with an apparent ED_{50} of ~0.05 μM, in the IVS2-705 background (Fig. 5B, lanes 10–15). In contrast, the tcAS oligonucleotide (identical in sequence to 2′-*O*-Me-PS-rAS, except for the additional 3′-terminal dA, which is partly cleaved off in serum; Fig. 3B) elicited a strong correction of β-globin mRNA splicing in both mutant backgrounds (Fig. 5A and B, lanes 4–9). In HeLa-654 cells, up to ~70% of β-globin mRNA could be spliced correctly and the apparent ED_{50} was ~0.03 μM. Correct splicing was more or less complete in HeLa-705 cells and the apparent ED_{50} was <0.01 μM.

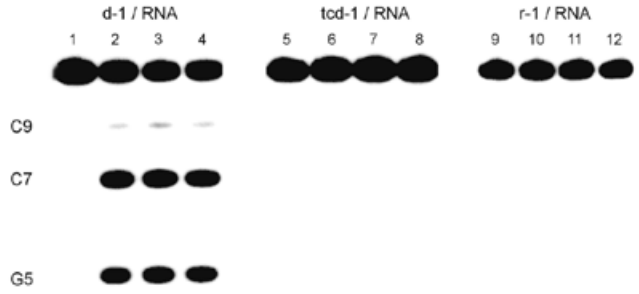

Figure 4. Autoradiogram of a 20% denaturing polyacrylamide gel showing the RNase H mediated cleavage of r(AACUGUCACG) by d-1 (lanes 1–4), tcd-1 (lanes 5–8) and r-1 (lanes 9–12) at 1, 30, 120 and 300 min each.

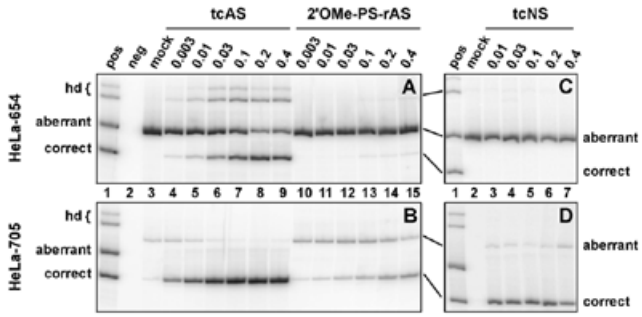

Figure 5. Correction of β-globin mRNA splicing after lipofection of tc-DNA oligonucleotide. Total RNA from HeLa cells expressing the human β-globin gene with either the IVS2-654 or IVS2-705 mutation, which result in the inclusion of an aberrant exon between the normal exons 2 and 3, was subjected to RT–PCR with primers in exons 2 and 3. Concentrations (μM) of the transfected oligonucleotides are indicated above the lanes. pos, RT–PCR products from cells expressing both the aberrant IVS2-654 and correct β-globin mRNA; neg (lanes 2 in **A**, **B** and **D**), RT–PCR with RNA from a mouse cell line not expressing β-globin; mock (lane 2 in **C**, lane 3 in A and B), RT–PCR with RNA from cells transfected with empty liposomes; hd, heteroduplexes between aberrant and correct RT–PCR products. Note that the aberrantly spliced product is longer in HeLa-705 than in HeLa-654 cells.

To test for the specificity of the observed antisense activity, we synthesized a control tc-DNA oligonucleotide, tcNS. This oligonucleotide lacks complementarity to the cryptic 3′-ss, but contains the sequence CCTTACCT whose complement is similar to the consensus sequence for 5′-ss, AG'GURAGU (R = A or G). Indeed, oligonucleotide tcNS did not correct the aberrant β-globin mRNA splicing in HeLa-654 cells (Fig. 5C), indicating that a six-base complementarity is insufficient to elicit antisense effects (Table 3). However, a strong correction of β-globin mRNA splicing was observed in HeLa-705 cells (Fig. 5D). Inspection of the sequences revealed that, with the IVS2-705 mutation, oligonucleotide tcNS and the mutant 5′-ss can form 11 bp separated by a single bulged nucleotide (Table 3).

In view of its less hydrophilic nature, it seemed possible that tc-DNA could also enter cells and elicit antisense effects without the help of transfection reagents. When this was tested in the same concentration ranges as used for the experiments in Figure 5, no correction of β-globin mRNA splicing could be observed (data not shown). However, with oligonucleotide concentrations of 10 or 40 μM, oligonucleotide tcAS, but not 2′-*O*-Me-PS-rAS, induced correct β-globin mRNA splicing in both HeLa-654 and HeLa-705 cells (Fig. 6). In HeLa-705 cells, a weak antisense effect could be observed down to 2 μM of

Table 3. Complementarities of oligonucleotide tcNS to IVS2-654 and IVS2-705 sequences

	IVS2-654	IVS2-705
wild-type	UUCUGGGUUAAG'GCAAUAGCAAUA •• • •• • • cTgACTCTC'CATTCCCAT	AUUGUAACUGAU'GUAAGAGGUUUC • •• ••••• ••• cTgACTCTC'CATTC-CCAT
mutant	UUCUGGGUUAAG'GUAAUAGCAAUA •• •••• • • cTgACTCTC'CATTCCCAT	AUUGUAACUGAG'GUAAGAGGUUUC • ••• ••••• ••• cTgACTCTC'CATTC-CCAT

The upper strand shows the relevant pre-mRNA sequence; apostrophes indicate the 5′-ss; the nucleotides changed by thalassemic mutations are shown in bold; and base complementarities are indicated by dots.

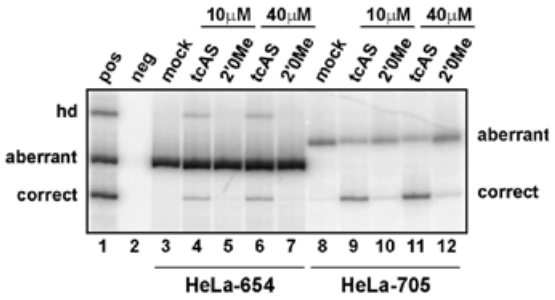

Figure 6. Correction of β-globin mRNA splicing after co-incubation (without liposomes) of tc-DNA oligonucleotide. Total RNA from HeLa-654 and HeLa-705 cells was subjected to RT-PCR with primers in exons 2 and 3. Concentrations (μM) of the transfected oligonucleotides are indicated above the lanes. pos, neg, mock and hd, as in Figure 5.

oligonucleotide tcAS (data not shown). Thus, tc-DNA can penetrate cells even in the absence of liposomal tranfection reagents, although higher concentrations are required to obtain antisense effects.

DISCUSSION

High affinity and selectivity in complementary RNA recognition is a prerequisite for successful antisense applications. tc-DNA shares both of these features. In the past, we focused on the synthesis and biophysical characterization of tc-oligonucleotides ≤10 nt in length. Such oligonucleotides show increases in T_m in the range of 1.9–2.4°C compared with RNA. We were pleased to find that now even longer oligonucleotides containing uninterrupted stretches of up to 17 tc units can be synthesized with the same efficiency. Furthermore, such oligomers show enhanced RNA-binding properties relative to 2′-*O*-alkyl-RNA or to morpholino antisense oligonucleotides (26), and comparable RNA binding properties with respect to the hexitol DNA analog HNA (15), or the N3′→P5′ phosphoramidates (27,28). A comparison with the LNA shows that the average increase in T_m/mod. is higher in the case of LNA (17,18,29). However, it appears that all-LNA sequences ≥13 nt in length tend to self-aggregate, thus inactivating themselves for RNA recognition (30). This seems not to be the case in tc-DNA and represents a net advantage.

We found earlier that tc-DNA sequences containing the bases adenine and thymine are stable against the 3′-exonuclease snake venom phosphodiesterase (20). Thus, it was not surprising to find that tc-DNA resists the endo- and exonucleases present in heat deactivated FCS. This test has often been used in the past for mimicking tissue culture-like nuclease conditions. The enhanced stability is the direct result of the structural changes at the centers C(3′) and C(5′). The fact that the last natural unit in the oligonucleotide tcAS is rapidly cleaved is an indication that the structural changes in tc-DNA inhibit the catalytic step in phosphodiester hydrolysis, and not the binding step by the enzymes. As a consequence of the inherent high biostability of tc-DNA the use of corresponding phosphorothioates is not necessary. This may help in reducing non-antisense effects and toxicity. Phosphorothioate groups are absolutely necessary in the case of DNA, and are of advantage in the case of 2′-*O*-alkyl-RNA in order to preserve stability in cellular environments. A more stringent test for the biostability of tc-DNA in fresh serum or *in vivo* will, however, be necessary in the next phase of evaluation.

Most of the sugar-modified antisense oligonucleotides are not able to induce RNase H activity in complex with RNA. Especially analogs that emulate RNA structure (RNA analogs) as, e.g., 2′-*O*-alkyl-RNA, LNA (30), HNA (31) and the N3′→P5′ phosphoramidates (28) are virtually unable to activate RNase H. Notable exceptions are the arabino-NA and the 2′-fluoro-arabino-NA (32), as well as the newly introduced analog CeNA (33,34). RNase H binds to DNA and RNA duplexes and, in some cases, to hybrids between oligonucleotide analogs and RNA, but these complexes are catalytically inactive. It is known that RNase H activity strongly depends on minor groove interactions (35). Thus, the size and shape of the minor groove and with this the conformational preference of the oligonucleotide analog are the determining factors for catalytic activity. The structural preference for an A-type double helical conformation in duplexes with DNA and with itself classifies tc-DNA as a structural RNA analog and thus explains its inability to elicit RNase H activity.

This inability to support RNase H-mediated RNA degradation means that an antisense activity would have to act by other means, e.g. by repressing cytoplasmic translation or by affecting nuclear events such as pre-mRNA splicing. Using an established system for measuring nuclear antisense effects (23,24), we were able to show a very significant correction of splicing of aberrantly spliced thalassemic β-globin pre-mRNAs in HeLa cells (Fig. 5). The correction obtained at the lowest dose of tcAS tested (0.003 μM) is comparable with that obtained with 0.2–0.4 μM of 2′-*O*-Me-PS-rAS containing the identical antisense sequence. This means that tc-DNA is approximately 100 times more efficient than 2′-*O*-Me-PS-RNA. In this respect, its efficiency seems comparable with morpholino and PNA oligonucleotides which showed a similar degree of superiority to both 2′-*O*-Me-PS and 2′-*O*-methoxyethyl-PS oligonucleotides in a similar test system, but using scrape loading to introduce the oligos into cells, since morpholino derivatives cannot be transfected with liposome reagents (36,37).

That tc-DNA may be equivalent to morpholino or PNA oligonucleotides is also suggested by the fact that antisense effects were observed even in the absence of transfection reagents (Fig. 6). With morpholino or PNA oligonucleotides, such liposome-free transfections could also be obtained, albeit with low efficiency; for PNA, the efficiency was increased by attaching one to four lysine residues (36,37). Here, the only

treatment consisted of a thorough mixing of tc-DNA with the cell culture medium.

Although the antisense effects of tc-DNA without lipofection were only seen at considerably higher concentrations, the direct transfection efficiency can probably still be improved. If this is the case, tc-DNA antisense oligonucleotides should be promising reagents for therapeutic *in vivo* applications. For this, however, animal toxicity studies would also have to be carried out.

Considering the strength of the observed nuclear antisense effects, it will be interesting to see if tc-oligonucleotides also inhibit mRNA expression either by affecting translation or by inducing mRNA degradation by means other than RNase H. If this turns out to be the case, then tc-DNA antisense oligonucleotides could be of similar use as morpholino oligonucleotides or double-stranded RNA interference (reviewed in 38) for studies of basic biological questions. Moreover, this would extend the therapeutic potential of tc-DNA beyond applications where alternative splicing needs to be modulated.

Our observations with the control oligonucleotide tcNS allow us to draw first conclusions concerning the specificity of tc-DNA antisense oligonucleotides in cultured cells. The potential for 6 contiguous bp in the case of the IVS2-654 5′-ss was insufficient. In contrast, the potential for 8 contiguous bp separated from 3 further bp by a single bulged nucleotide at the IVS2-705 5′-ss was sufficient to elicit an antisense effect similar to that seen with oligonucleotide tcAS (Fig. 5). However, it must be noted that the 5′-ss created by the IVS2-654 and IVS2-705 mutations are more efficient targets for antisense oligonucleotides than the cryptic 3′-ss targeted by oligonucleotide tcAS (24).

tc-oligonucleotides show improved pairing selectivity to their target, compared with RNA or DNA (Table 2). However, with longer sequences an overall increased thermal stability of a matched duplex is typically also associated with an overall increased stability of a mismatched duplex. This is a severe handicap in the application of high-affinity antisense probes. One way out could consist of the design of oligonucleotides that are as short as possible in order to discriminate mismatches more efficiently, but that are still long enough to maintain target specificity.

Our experiments imply that tc-DNA antisense sequences of 11 nt (or shorter) may be sufficient to obtain antisense effects *in vivo*. This compares favorably with the longer antisense sequences commonly used for other types of oligonucleotides. The finding is also in agreement with the high melting temperatures observed for tc-DNA–RNA hybrids (Table 2). For potential applications, this shorter length requirement would also translate into lower costs and higher yields, and potentially even better cellular transfer properties, of the oligonucleotides.

The question of ss specificity must still be analyzed in more detail. The control oligonucleotide tcNS was designed to fit an unrelated 5′-ss. Its efficient antisense effect in HeLa-705 cells implies a certain risk when targeting sites with long consensus sequences. Perhaps 5′-ss, with their rather extensive sequence conservation, will show too much cross-reactivity. In contrast, 3′-ss, with their shorter consensus sequence, may represent more specific targets. As an alternative in the case of 5′-ss, one could shift the antisense sequence more into the less conserved exonic part. Future analyses should show precisely what the minimum length requirement is and how cross-reactivity, potentially translating into unwanted side-effects, can be avoided.

In conclusion, this study establishes that tc-DNA oligonucleotides can very efficiently mediate antisense effects that are of considerable potential both for medical applications and studies of basic biology.

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