Triple Helices of Optimally Capped Duplex DNA (σ-DNA) with Homopyrimidine DNA and RNA at Neutral pH

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Dedicated to Prof. Dr. *Frank Seela* on the occasion of his 60th birthday

An optimally capped duplex DNA (σ-DNA) was synthesized in which the cytosines in the homopyrimidine strand of σ-DNA **1** were replaced by 5-methylcytosine leading to σ-DNA **2**. Although only involving 13 base pairs, this modification resulted in very high melting temperatures above 90°. In addition, σ-DNA **2** was able to form triple helices with the corresponding homopyrimidine DNA or RNA even at neutral pH. This opens up the possibility to use σ-DNA in a triple-helix approach to modulate gene expressions on the level of the translation process.

1. Introduction. – Double-stranded DNA is able to align a third strand of DNA or RNA in its major groove to form triple-helix motifs [1][2]. In a Pyr·(Pur·Pyr) motif, the third strand is bound *via Hoogsteen* H-bonds (*Fig. 1*). Such an alignment requires the protonation of the C residues in the third strand. Although restricted to homopyrimidine or homopurine sequences, the triple-helix approach is of interest with respect to the specific control of gene expressions.

Binding of a third strand into the major groove of double-stranded target DNA can prevent the binding of transcription factors and hence, prevent the initiation of the transcription process of a gene of interest. This strategy, also dubbed the anti-gene approach, was pursued by several groups [3][4].

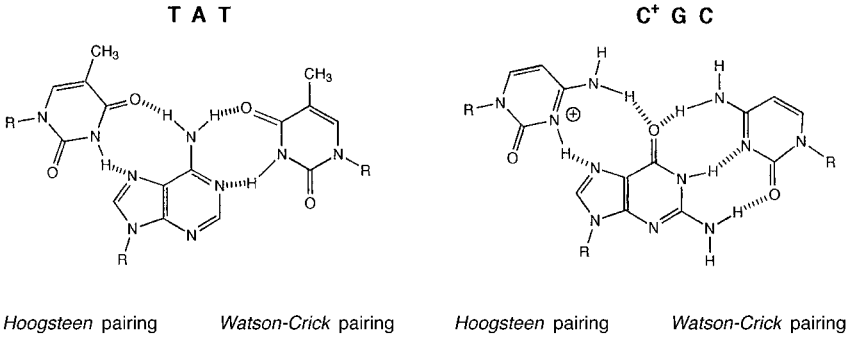

Fig. 1. Hoogsteen *pairing in the Pyr·(Pur·Pyr) mode*

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An alternative strategy based on triple-helix formation and aiming at the inhibition or modulation of the translation process, was developed by *Kool* [5]. Application of properly designed circular homopyrimidine constructs can be used to bind single-stranded homopurine sequences. One homopyrimidine region of the circular structure is engaged in *Watson-Crick* pairing and the other one in *Hoogsteen* pairing, resulting in a tight overall binding of the circular structure to the homopurine strand, *e.g.* to an envisaged m-RNA, and thus preventing translation.

Recently, we have synthesized a variant of cyclic DNA that represents in essence an optimally capped duplex DNA [6][7]. We have dubbed this type of DNA as σ-DNA to distinguish it from 'dumbbell' DNA. We were able to demonstrate that such a σ-DNA represents a very stable mimic of long double-stranded B-DNA showing very high *Watson-Crick* melting temperatures. If one strand of the σ-DNA consisted of a homopyrimidine sequence and the other one of a homopurine sequence, we were able to align a third homopyrimidine strand in the major groove of the σ-DNA, resulting in a strong *Hoogsteen* binding. As expected, this binding was pH-dependent and decreased with increasing pH. At pH 7.0, no *Hoogsteen* binding with DNA was observed, and with RNA as third strand, the *Hoogsteen* melting temperature was only 17°.

2. Results and Discussion. – The aim of this work was to modify σ-DNA in such a way that it is able to align a homopyrimidine RNA strand in a *Hoogsteen* mode, even at neutral pH, with a binding efficiency comparable with the binding of a complementary single-stranded DNA fragment to the same target RNA strand, a strategy which one would apply to standard antisense approaches.

Our earlier results have indicated that the stability of the *Hoogsteen* binding of RNA to σ-DNA is higher than that of the corresponding DNA at all pH values investigated [7]. This is in accordance with results published by *Roberts* and *Crothers*, who demonstrated that the stability of triple helices is dependent on the nature of the individual strands engaged in the triple-helix formation (DNA or RNA) [8]. Furthermore, these experiments had indicated that the stability of the *Hoogsteen* binding was dependent on the stability of the *Watson-Crick* duplex of the σ-DNA. The more stable the σ-DNA, the more stable the *Hoogsteen* interaction. Thus, the aim was to increase the stability of this *Watson-Crick* pairing to such an extent that it was able to bind single-stranded RNA even at neutral pH with decent efficiency.

The stability of cytosine · guanine base pairs can be increased by replacing the cytosine by 5-methylcytosine [9]. The 5-methylcytosine is a naturally occurring base which plays a role in the control of transcription in eucaryotes [10]. Thus, we hoped that σ-DNA **2** in which all cytosines of σ-DNA **1** were replaced by 5-methylcytosine would be so stable as to allow for binding of the single-stranded RNA fragment **4** *via Hoogsteen* mode with the desired efficiency at neutral pH. At the same time, the binding to the corresponding DNA fragment **3** was also investigated (*Fig. 2*).

The strategy for the synthesis of compounds **1** and **2** is outlined in *Fig. 3*. The preparation is based on the cyclization of the linear precursors by a chemical ligation. In these linear precursors, backfolding of the overhanging ends guarantees close proximity between the phosphate group and the OH function for formation of the phosphodiester bond. The ligation can be performed starting either from the linear 5′- or the 3′-phosphorylated fragment with comparable efficiency [6]. For the synthesis of **1** and **2**,

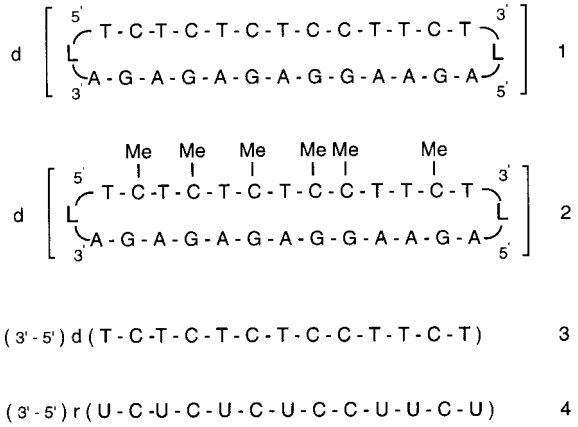

Fig. 2. *DNA and RNA sequences used in the melting experiments*

we started from the 3′-phosphates employing a disulfide linker to the support, which yields, after reduction of the disulfide bond, *via* spontaneous elimination, directly the desired 3′-phosphorylated fragment [11]. The chemical ligation was performed employing *N*-[3-(dimethylamino)propyl]-*N*′-ethylcarbodiimide hydrochloride (EDC), and a high conversion to the circular DNA **1** and **2** was achieved. The reaction was performed with the crude unpurified linear fragments.

The linker unit consists of a hexaethylene-glycol spacer containing two phosphate groups at its termini. This linker proved ideal for bridging the terminal base pairs without causing conformational strains in B-DNA. At the same time, this linker is short enough to restrict efficiently the conformational flexibility of the connected nucleotide ends [12].

The results of the melting experiments are summarized in the *Table*. Substitution of the cytosines by 5-methylcytosines resulted as expected in a greater stability of the *Watson-Crick* duplex, and this at all pH values investigated. Hence, the melting of **2** is generally above 90° although it involves only 13 base pairs. At the same time, the *Hoogsteen* melting temperatures of *σ*-DNA **2** with the single-stranded DNA fragment **3** and the RNA fragment **4** were higher in comparison with the corresponding ones with *σ*-DNA **1**. At all pH values, this increase in the *Hoogsteen* binding was more pronounced for DNA fragment **3** as the third strand as compared to the RNA fragment **4** as the third strand.

In *Fig. 4*, we have compared the melting of *σ*-DNA **2** with the homopyrimidine DNA **3** and the corresponding RNA sequence **4** from pH 5.0 to pH 7.5. Over this pH range, the binding of *σ*-DNA **2** to the RNA **4** as the third strand in the *Hoogsteen* mode was stronger than the binding of **2** to the DNA **3**. This difference became smaller with increasing pH value. Nevertheless, *Hoogsteen* binding was still observed in both experiments at neutral pH. At pH 7.0, the *Hoogsteen* melting temperature with RNA (31.6°) was comparable with the *Watson-Crick* melting of a duplex composed of the RNA sequence **4** and the complementary DNA sequence (5′-3′)d(A-G-A-G-A-G-A-G-G-A-A-G-A) which showed under identical conditions a melting temperature of

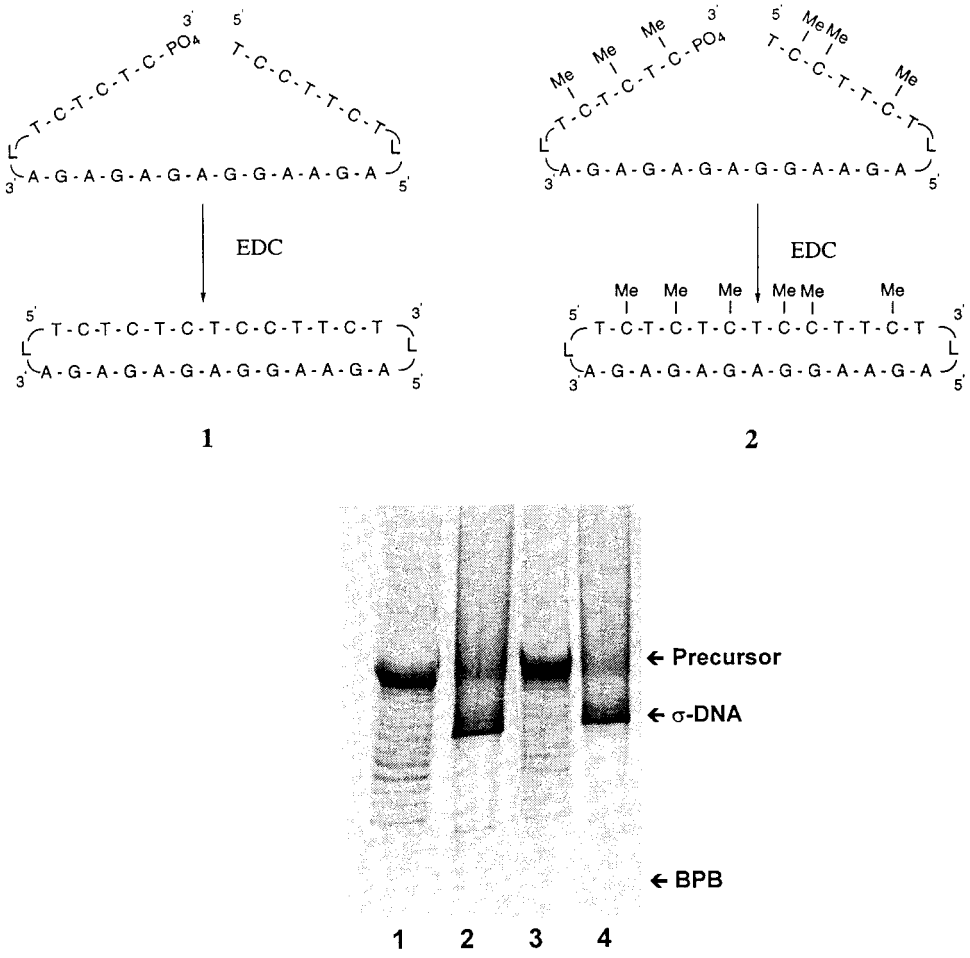

Fig. 3. *Cyclization strategy for the synthesis of the σ-DNA molecules* **1** *and* **2**, *and PAGE* (polyacrylamide-gel electrophoresis) *of the crude reaction mixtures. Lane 1:* crude linear precursor for **1**; *Lane 2: cyclization reaction leading to* ***1***; *Lane 3:* crude linear precursor for **2**; *Lane 4:* cyclization reaction leading to **2**.

Table. *Results of Duplex and Triplex Melting Experiments with Sequences* **1**–**4**. Conditions as in *Figs. 4* and *5*.

σ-DNA	*Hoogsteen* strand	pH 5.0		pH 5.5		pH 6.0		pH 6.5		pH 7.0		pH 7.5	
		melt 1	melt 2	melt 1	melt 2	melt 1	melt 2	melt 1	melt 2	melt 1	melt 2	melt 1	melt 2
1	**3**	54.2	83.4	46.3	85.3	32.3	86.8	20.6	87.4	–	87.4	–	87.5
2	**3**	64.7	90.0	55.5	> 90	43.3	> 90	34.3	> 90	29.1	> 90	26.4	> 90
1	**4**	67.6	83.8	57.8	85.8	42.4	86.6	29.2	87.7	–	87.2	–	87.3
2	**4**	74.2	> 90	63.2	> 90	49.0	> 90	38.8	> 90	31.6	> 90	28.1	> 90

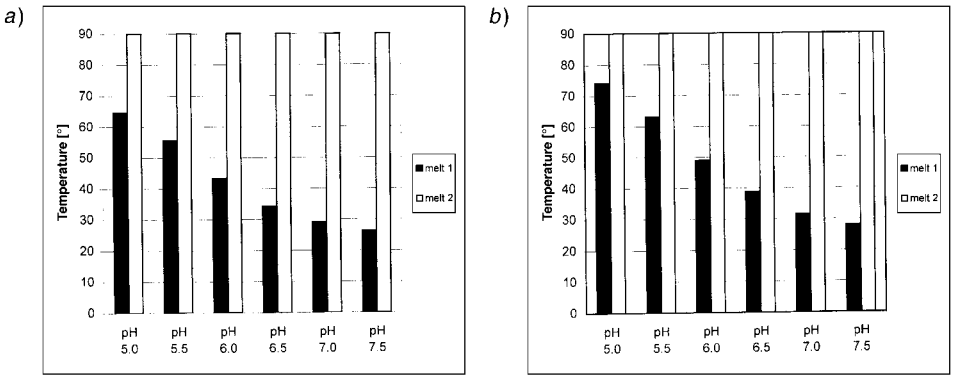

Fig. 4. a) *pH Dependency of the melting points of σ-DNA* **2** *and single-stranded DNA* **3** *as third strand.* b) *pH Dependency of the melting points of σ-DNA* **2** *and single-stranded RNA* **4** *as third strand. Melt 1: Hoogsteen* melting temperature; *Melt 2: Watson-Crick* melting temperature. Conditions: 1.0 μM DNA, 100 mM NaOAc/AcOH (pH 5.0–7.5); 1 mM EDTA.

30.7°. This situation is representative of the normal antisense situation with a linear unmodified DNA fragment binding to the complementary RNA.

In *Fig. 5*, we have illustrated the difference of the *Hoogsteen* melting temperatures applying either *σ*-DNA **1** or *σ*-DNA **2**. The stabilization effect obtained by substituting cytosine by 5-methylcytosine was clearly demonstrated.

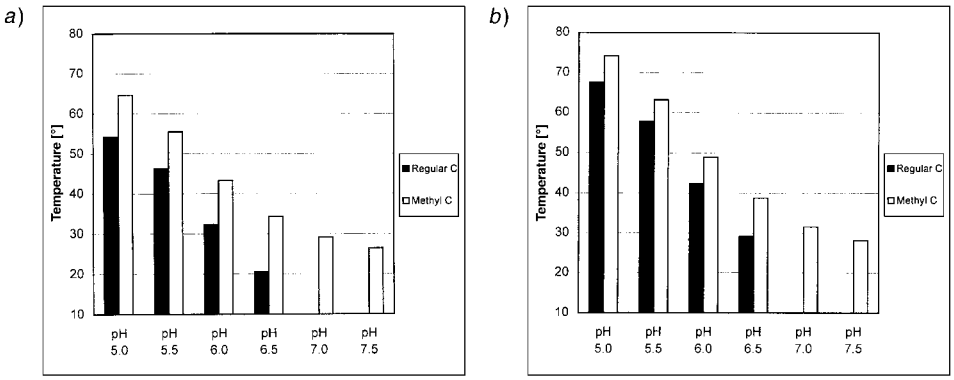

Fig. 5. a) *pH Dependency of the* Hoogsteen *melting temperatures of either σ-DNA* **1** *or σ-DNA* **2** *and DNA* **3** *as* Hoogsteen *strand.* b) *pH Dependency of the* Hoogsteen *melting temperature of either σ-DNA* **1** *or σ-DNA* **2** *and RNA* **4** *as third strand*. Conditions as in *Fig. 4*.

3. Conclusion and Outlook. – We were able to demonstrate that triple-helix formation of optimally capped duplex DNA (*σ*-DNA) is possible under neutral conditions with DNA, as well as with RNA, as the third strand. This was achieved by substitution of the cytosines in *σ*-DNA **1** by 5-methylcytosine leading to *σ*-DNA **2**. The *Hoogsteen* melting of *σ*-DNA **2** with the corresponding homopyrimidine RNA sequence **4** at neutral pH was in the same range as the *Watson-Crick* melting of the corresponding linear DNA·RNA hybrid. Hence, *σ*-DNA could be used in an alternative approach to inhibit or modulate the translation of mRNA.

Apart from the hexaethylene-glycol linker, σ-DNA consists of only naturally occurring bases and is unmodified at the phosphodiester internucleotide linkages. Thus, possible breakdown products should not lead to cell toxicity. Due to the cyclic structure of σ-DNA, such a degradation could only be initiated by double-strand specific endonucleases.

In addition, the synthesis of the σ-DNA was achieved with high efficiency by a chemical ligation of the crude phosphorylated linear precursor molecules.

Experimental Part

1. *General*. All solvents were of highest purity available. The 1,4-dithioerythritol (= *erythro*-1,4-dimercaptobutane-2,3-diol; DTE), *N*-[3-(dimethylamino)propyl]-*N*′-ethylcarbodiimide hydrochloride (= EDC), undecane, and *Stains-all* were from *Fluka*, $Bu_4NF \cdot 3\,H_2O$ from *Aldrich*, and *NAP-10-Sephadex* columns from *Pharmacia*. All DNA and RNA syntheses were performed on an *ABI-394* synthesizer; the different solns., supports, and amidites for DNA synthesis were from *ABI*. The RNA building blocks, *i.e.*, 5′-(dimethoxytrityl-2′-[(*tert*-butyl)dimethylsilyl]-protected ribonucleoside (2-cyanoethyl diisopropylphosphoramidites), were from *Milligen*. 30% Sat. NH_3/EtOH soln. was prepared by bubbling NH_3 gas slowly through 30 ml of ice-cooled EtOH (*Aldrich*; H_2O max. 5 ppm) for 2 h. The UV-absorption/melting curves were run on a *Cary-3-UV* photometer. The hexaethylene glycol building block was synthesized as described in [7].

2. *DNA Fragment* **3** was prepared on controlled-pore glass (CPG; *Sigma*) as solid support [13] applying a 10-fold excess of 2-cyanoethyl phosphoramidites [14] and a 130-fold excess of 1*H*-tetrazole. The workup was done using our standard technology [15].

3. *RNA Fragment* **4** was synthesized on a 1-μmol scale using per coupling a 150-fold excess of 2′-*O*-[(*tert*-butyl)dimethylsilyl]-protected phosphoramidite [16] and 150-fold excess of 1*H*-tetrazole with a coupling time of 15 min and the standard RNA cycle [17]. The workup was performed under sterile conditions. The support was treated with 1 ml of sat. NH_3/EtOH soln. at 70° for 3 h. The NH_3 soln., including 1 ml of washing soln. of the support with EtOH/H_2O 6 : 4, was evaporated on a *Speed-vac* concentrator. After addition of 0.5 ml of fresh 1M Bu_4NF/THF, the pellet was vortexed for 16 h at r.t. until a soln. was obtained. Then 0.4 ml of 0.5M NH_4OAc soln. (pH 7.0) was added and the soln. evaporated to 300 μl and desalted by a *NAP-10* filtration. The fragment was purified by prep. 20% polyacrylamide-gel electrophoresis (PAGE) under denaturing and sterile conditions [17].

4. *σ-DNA* **1** was synthesized in two steps. Firstly, the 3′-phosphorylated precursors were synthesized, and in a second step, these were cyclized to yield **1**. For the synthesis of the 3′-phosphates, we used a disulfide linker as described in [11] but employing a sarcosine-modified CPG for the attachment of the linker unit. Preparation of the linear fragment was performed starting with 1 μmol of the modified support. When the linker had to be introduced, coupling with the appropriate phosphoramidite was carried out applying a standard cycle. After complete assembly, the $(MeO)_2Tr$ group was removed. Further deprotection and release as 3′-phosphate was by treatment with 1 ml of 0.2M DTE in conc. NH_3 soln. for 2 h at 70°. After evaporation of the soln. on a *Speed-vac* concentrator, the fragments were precipitated after addition of 100 μl of H_2O, 200 μl of dioxane, and 600 μl of THF. Part of the crude material was analyzed by anal. PAGE to assess the performance of the synthesis (*Fig. 3*).

The cyclization of the linear 3′-phosphorylated fragment with EDC to yield the σ-DNA **1** was performed in 1 ml of 50 mM MES (morpholine-4-ethanesulfonic acid) pH 6.0, 20 mM $MgCl_2$, and 400 mM EDC containing 200 mmol (80 *OD*) of the crude linear precursor. After 5 h, the crude mixture was desalted on a *NAP-10* column and purified by prep. PAGE. The purity was assessed by anal. PAGE after staining with *Stains-all*. MALDI-TOF-MS confirmed the correct molecular mass of the fragment.

σ-DNA **2** was prepared in the same way as **1**, except that the 2′-deoxy-5-methylcytidine phosphoramidite was used as building block instead of the corresponding 2′-deoxycytidine building block.

5. *Melting Curves*. The UV absorbance was measured at 260 nm at 95°, and the following extinction coefficients were employed: A 15000; C 7500; G 12500; T 8500 [18]. Each fragment (1 nmol) was dissolved in 1 ml of 1 mM EDTA and 100 mM sodium acetate (pH 7.5) adjusted to the corresponding pH with AcOH. After transfer into the cuvettes, the solns. were degassed by sonications at 90°. Then a layer of 0.1 ml of undecane was added, and the cuvettes were closed by stoppers. UV-Absorption/melting curves were measured against cuvettes containing the buffer alone. The heating program was from 10 to 95° with a 0.5° increase per min and from 95 to 10° with 0.5° per min. Each melt was performed at least twice, and the given melting temp. is the average of the two measurements. Only the melting temp. from the heating run was used for the calculation. Melting temps. were calculated from the first derivative of the raw data.

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