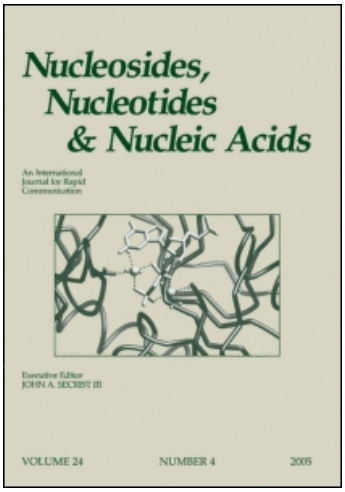

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SYNTHESIS OF A POLYAMINOOLIGONUCLEOTIDE COMBINATORIAL LIBRARY

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ABSTRACT: A new method of synthesis of phosphoramidite of 2'-deoxycytydine carrying a protected spermine moiety at *N*-4 position is described. A model oligodeoxyribonucleotide containing the specified nucleoside unit has been synthesised. A synthesis of a polyaminooligonucleotide combinatorial library was carried out. The analysis of the above library clearly shows that the presence of spermine moieties in oligodeoxyribonucleotides increases stability of their duplexes.

INTRODUCTION

In the past three decades there has been considerable interest in naturally occurring polyamines such as putrescine, spermidine or spermine, because of their essential role in cell processes and potential in cancer chemotherapy[1-4]. Natural as well as modified polyamines are well known to stabilise nucleic acids tertiary structures[2,5-8]. Recently, oligonucleotides carrying polyamine residues of different structure covalently linked at various positions of oligonucleotide chains were described[9-16]. These modifications were found to stabilise duplex and triplex structures of nucleic acids. This property of polyamines might be applied in antigen therapy. Therefore, the important area in research of polyamine-DNA interactions relies on development of new methods of synthesis of polyamines covalently linked to deoxyribonucleic acids. Furthermore, a development of new methods for investigation of influence of polyamines upon a thermal stability of duplex and triplex DNA structures becomes very important.

Polyamine moieties were tethered to oligonucleotides using different methods. Tung *et al.*[9] attached spermine residue *via* a linker to a 5'-end in a post-solid phase

synthetic step. Prakash *et al.*[10] conjugated spermine molecule to the *N*-4 of the 5-methyl-2'-deoxycytidine and then incorporated this modified nucleoside into oligonucleotides making use of the phosphoramidite chemistry. Nara *et al.*[11] synthesized a novel 2'-deoxyuridine analogue with *syn*-norspermidine at *C*-5 position and introduced to heptadecadeoxynucleotides using the phosphoramidite solid phase synthesis. Schmid *et al.*[12] presented a synthesis of a modified 11-mer oligodeoxyribonucleotide containing spermine residues linked to the *C*-2 position of deoxyinosine units, but spermine molecules were attached in a post-synthetic deprotection step using a fully protected 2-fluoro-2'-deoxyinosine phosphoramidite in the solid phase synthesis. Bigey *et al.*[13] employed spermine as a linker between 5'-end of an oligonucleotide and a tris(methylpyridiniumyl)porphyrinat-manganese(III) motif in a post-solid phase synthetic step. Finally, Sund *et al.*[14] reported coupling of a new *C*-branched spermine derivative to 5'- or 2'-hydroxyl groups *via* a phosphate bridge in a synthesis of oligonucleotides. In all reports the positive impact of polyamines on a thermal stability of DNA double and/or triple helices was observed.

We propose to name oligonucleotides with covalently attached polyamine moieties as *polyaminooligonucleotides*. In order to carry out systematic studies on polyaminooligonucleotides we have undertaken preparation of phosphoramidites of deoxynucleosides with polyamines moieties linked directly to base residues. In this report a new synthesis of deoxycytidine modified at *N*-4 position with a spermine residue and its introduction to oligonucleotides is presented. In the same time, we propose to investigate polyaminooligonucleotides using a combinatorial chemistry approach recently developed in our laboratory[17-19].

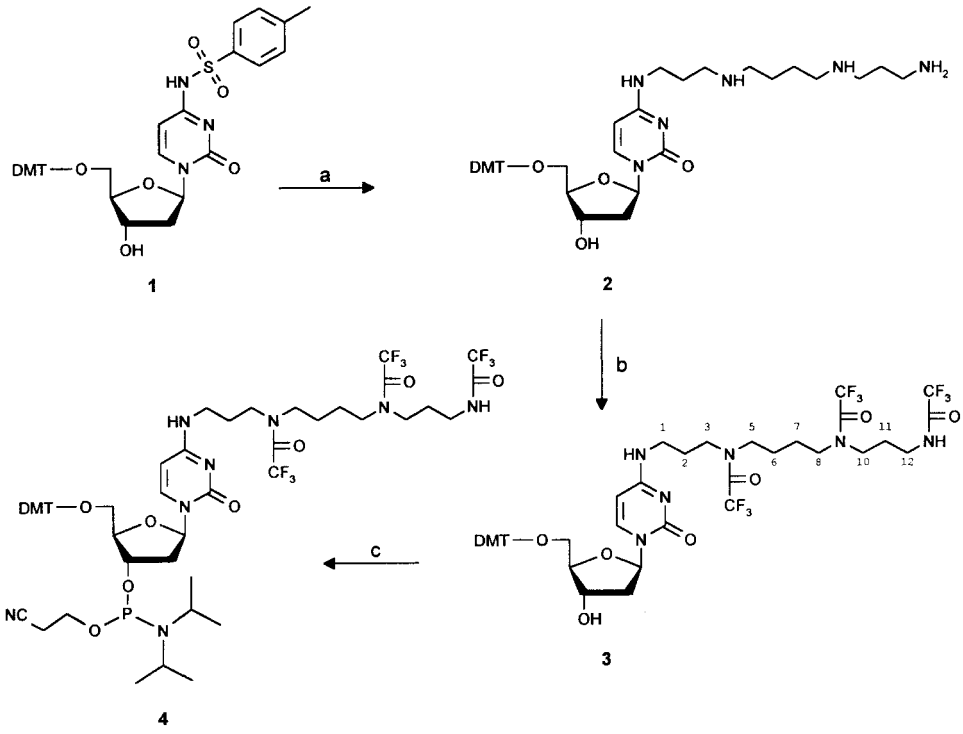
RESULTS AND DISCUSSION

We decided to synthesise oligonucleotides with deoxycytidine units modified at *N*-4 position with the spermine residue. An attachment of a substituent at *N*-4 position of deoxycytidine does not disturb Watson-Crick base pairing[20-22]. On the other hand, oligonucleotides with 5-methyldeoxycytidine units carrying spermine moieties at *N*-4 positions were found to stabilise triplex DNA complexes[10]. The latter ones were obtained from deoxythymidine as a substrate[10].

In order to synthesise oligonucleotides with deoxycytidine units modified at *N*-4 position with polyamine residues an appropriate fully protected phosphoramidite was prepared. A reaction described by us previously, namely the conversion of 4-*N*-arylsulfonylcytosine residues with primary amines into 4-*N*-aminoalkyl substituted cytosine derivatives[20] was applied. Thus, 4-*N*-*p*-toluenesulfonyl-5'-*O*-dimethoxytrityl-2'-deoxycytidine[20,21] (**1**) was reacted with spermine (SCHEME 1). The TLC analysis showed that the reaction went to completion after overnight treatment of **1** with tenfold excess of spermine in pyridine at 70 °C. Trifluoroacetylation of the product using ca sevenfold molar excess of trifluoroacetic anhydride in pyridine at room temperature proceeded smoothly in 5 min to give 5'-*O*-dimethoxytrityl-4-*N*-[tris(*N,N',N''*-trifluoroacetyl)-4,9,13-triazatridecane-1-yl]-2'-deoxycytidine (**3**), which was obtained after silica gel chromatography in 60% overall yield. Its structure was confirmed by ^{1}H NMR analysis. In order to obtain a phosphoramidite of **3** in the final step of the synthetic route, pure **3** was subjected to phosphitylation with bis(*N,N*-diisopropylamino)(2-cyanoetoxy)phosphine in the presence of tetrazole. The reaction ended after 1 h and the purification gave the desired product, 3'-phosphoramidite of 5'-*O*-dimethoxytrityl-4-*N*-[tris(*N,N',N''*-trifluoroacetyl)-4,9,13-triazatridecane-1-yl]-2'-deoxycytidine (**4**), in high yield (SCHEME 1). The ^{1}H and ^{31}P NMR spectra of phosphoramidite **4** corroborated its structure.

The phosphoramidite **4** was then employed in order to check its synthetic performance. A synthesis of a model 14-mer oligodeoxyribonucleotide d-TTTTTTC^{Sp}TTTTTTT was carried out. The coupling efficiency of **4** was the same as of the standard dC phosphoramidite. The deprotection under standard conditions using concentrated aqueous ammonia at 55 °C overnight allowed to remove all protecting groups including trifluoroacetyls[10].

We decided to take the advantage of the approach of synthetic oligonucleotide combinatorial libraries (SOCL) developed recently in our laboratory[17-19] to study properties of polyaminooligonucleotides. A highly cross-linked polystyrene support with uniform beads of 50 μm diameter and average pore size ca 1000 Å is well suited for preparation of dispersed SOCLs after appropriate functionalisation[17] and therefore, we used this support in the studies presented in this report.



SCHEME 1. Key: (a) spermine (10 equiv.) in pyridine, 70 °C, 17 h; (b) $(CF_3CO)_2O$ (5 equiv.) in pyridine, room temp., 5 min, 60% overall yield; (c) bis(*N,N*-diisopropylamino)(2-cyanoetoxy)phosphine (1 equiv.), tetrazole (1 equiv.) in CH_2Cl_2, room temp., 1 h, 81% yield.

A short oligonucleotide, an octamer d-CCCCTTTT was chosen as a first target sequence. This oligonucleotide was synthesised on the polystyrene support by the phosphoramidite approach and remained bound to the support after deprotection procedure (FIG. 1A). A linker between an oligonucleotide and a solid support allowing for the permanent anchorage of oligomer in a SOCL was used as described earlier[17].

Then, a small polyaminooligonucleotide combinatorial library was prepared on the polystyrene support. The synthesis was done using split-combine approach[23-25] leading to the SOCL in which each bead should carry an oligonucleotide of unique sequence (*one bead - one oligomer*). Thus, the polyaminooligonucleotide library with four randomised positions d-XXXXTTTT, where X is either deoxycytidine (C) or deoxycytidine modified

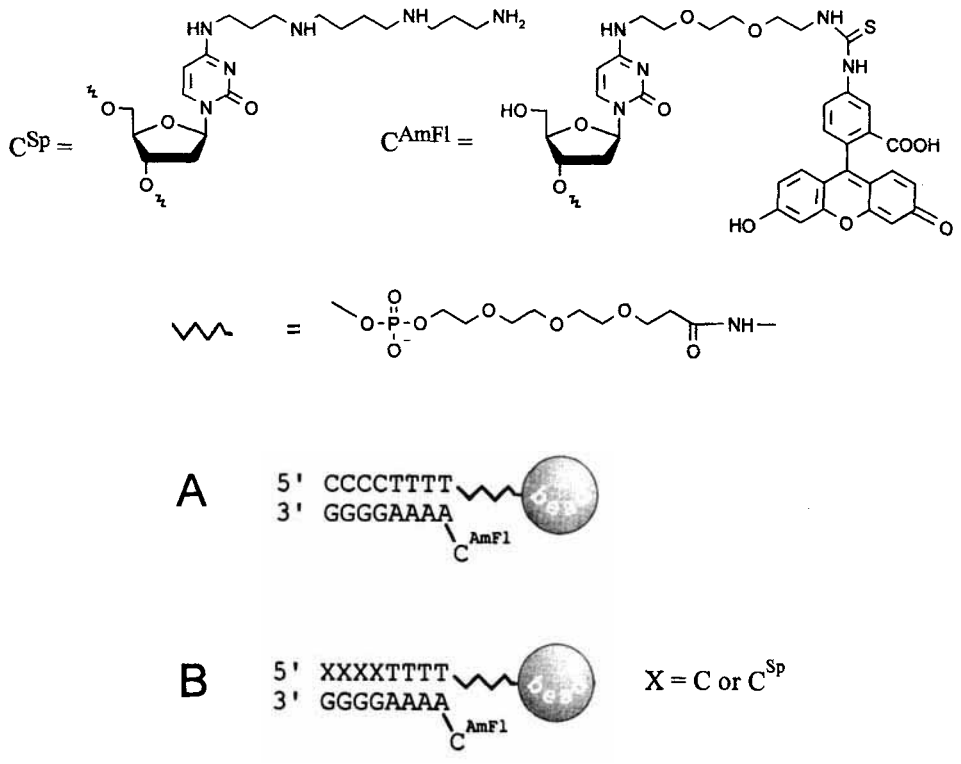


FIG. 1. Structure of (A) the polystyrene supported unmodified octamer and (B) the polyaminooligonucleotide combinatorial library hybridised with the fluorescently labelled oligonucleotide probe.

with the spermine residue (C^{Sp}) was made. The library elements have a structure shown in FIG. 1B. The slurry of the polystyrene solid support in anhydrous acetonitrile was handled at split steps to avoid losses of material as well as crashing of the support during the SOCL synthesis and analysis. As a result the library containing $2^4 = 16$ individual oligonucleotide elements was obtained.

Then, a complementary oligonucleotide to be used as a hybridisation probe, d-C^{AmFl}AAAAGGGG, containing at the 5'-end an extra deoxycytidine unit labelled with fluorescein was prepared as previously described[20]. The d-CCCCTTTT support (FIG. 1A) was hybridised with the fluorescein labelled probe under less stringent conditions (FIG. 2A, Experimental). The visual analysis using an epifluorescent microscope revealed that practically all beads were hybridised with the probe (FIG. 2A).

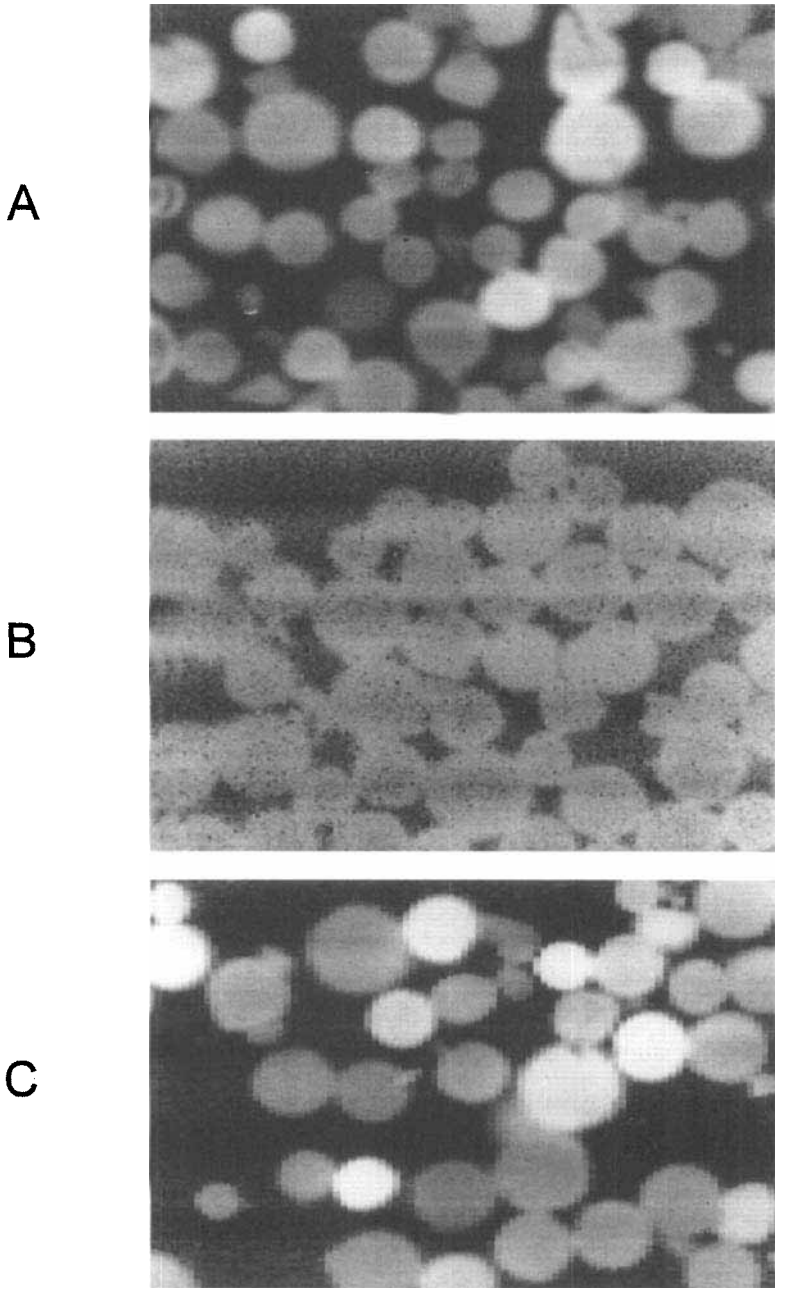


FIG. 2. The results o hybridisation of the labelled probe d-C^{AmFl}AAAAGGGG with: (A) the polystyrene supported octamer d-CCCCTTTT under less and (B) more stringent conditions; (C) the polyaminooligonucleotide SOCL under stringent conditions (Nikon Diaphot, magnification 200x).

Then, the more stringent conditions with the decreased concentration of the fluorescent probe and 5% of formamide were applied. This resulted in disappearing of fluorescence of beads of unmodified supported octamer (FIG. 2B).

However, when these more stringent conditions were applied to the polyaminooligonucleotide SOCL (FIG. 1B) brightly fluorescing beads could be easily found (FIG. 2C). This indicates unequivocally that introduction of the spermine residue into *N*-4 position of deoxycytidine unit(s) results in a strong improvement of stability of oligonucleotide complexes.

In our opinion it should be pointed out that a combinatorial chemistry approach opens a new route to study different properties of modified oligonucleotides and can substantially accelerate searching of unique structures of modified oligonucleotides not only of the type reported in this communication. Further studies concerning other modified oligonucleotides are in progress and will be reported elsewhere.

EXPERIMENTAL

General methods

All the solvents used in the reactions were purified and dried according to the earlier published procedures. Trimethylsilyl chloride - TMSCl (POCh, Poland), p-toluenesulfonyl chloride (Fluka), concentrated aqueous ammonia (Merck), 4,4'-dimethoxytrityl chloride (Chemipan, Poland), spermine (Fluka), trifluoroacetic anhydride (Merck), fluorescein isothiocyanate isomer I - FITC (Fluka) were used directly. Synthesis of bis(*N*,*N*-diisopropylamino)(2-cyanoetoxy)phosphine was carried out in our laboratory on the grounds of the well established procedure[26]. The organic extracts were dried over anhydrous sodium sulphate. Thin layer chromatography was performed on E. Merck pre-coated plates silica gel 60 HF_{254} in the following solvent systems: A - dichloromethane/methanol (9:1); B - dichloromethane/methanol (95:5); C - isopropanol-concentrated aqueous ammonia-water (7:1:2). Short column chromatography was performed on E. Merck silica gel H 60 in the following eluents: dichloromethane/methanol (95:5), dichloromethane/methanol (95:5) in 0.5% pyridine solution, ethyl acetate/triethylamine (9:1), isopropanol/concentrated aqueous ammonia/water (7:1:2). All solvent ratios are by volume. ^1H NMR spectra were recorded on a Varian Unity 300 NMR spectrometer operating at 299.9 MHz using tetramethylsilane (TMS) as an internal standard. ^{31}P NMR spectra were recorded on the same apparatus operating at 121.4 MHz with 85% ortophosphoric acid as an external standard. Oligonucleotides were synthesised with the following automated DNA synthesisers: the Gene Assembler Plus from Pharmacia-LKB (Sweden) and a home built system. NAP-25 columns (Pharmacia LKB) were used to separate oligonucleotides from lower molecular weight compounds (ammonia, salts etc.).

A highly cross-linked polystyrene support was kindly provided by Dr. Alex Andrus (Applied Biosystems, USA). Polystyrene functionalisation was performed as described earlier[16]. The inverted microscope Diaphot TMD (Nikon, Japan) with the high voltage mercury lamp (output power 100 W), filter B-A1EX470-490 for fluorescein was used in visual analysis of SOCLs. The microscope was equipped with a computer linked CCD TV camera (ELMO, Poland) for recording of pictures of analysed SOCLs.

5'-*O*-Dimethoxytrityl-4-*N*-[tris(*N,N',N''*-trifluoroacetyl)-4,9,13-triazatridecane-1-yl]-2'-deoxycytidine (3). To a solution of **1** (543 mg, 0.8 mmole) in anhydrous pyridine (10 mL) spermine (2.023 g, 8.0 mmoles) was added. The reaction flask was closed and kept in an oven at 70 °C overnight. The reaction was ended and the TLC analysis indicated formation of **2** with R_f(C) 0.1. Next, the solution was poured into water (20 mL). The aqueous layer was extracted twice with dichloromethane (30 mL). After each mixing of layers an emulsion was formed, so the mixture was centrifuged to separate layers. The organic phase was evaporated under reduced pressure. The oily residue of **2** (646 mg, ca 0.9 mmole) was dissolved in dry pyridine (10 mL) and then trifluoroacetic anhydride (644 μL, 4.5 mmoles) was added. An instant change of colour of the reaction mixture from yellowish to dark-yellow was noticed. The TLC analysis showed that the reaction went to completion in 5 min: R_f(B) 0.26. The solution was partitioned between water (30 mL) and dichloromethane (30 mL). The aqueous phase was extracted with dichloromethane (2 × 30 mL). The organic extracts were evaporated under reduced pressure and the residue was subjected to short column chromatography (CH_2Cl_2/MeOH, 95:5, 0.5% solution of pyridine) to obtain pure **3** as a yellowish foam: 498 mg, 60% overall yield. ^{1}H NMR ($CDCl_3$): δ (ppm) 7.79 (d, 1H, J 7.5 Hz, H-6), 6.77-7.41 (m, 13H, DMT aromatic), 6.26-6.34 (m, 1H, H-1'), 5.82-5.88 (m, 1H, OH-3'), 5.35-5.42 (m, 1H, H-5), 4.52 (m, 1H, H-3'), 4.05 (m, 1H, H-4'), 3.79 (s, 6H, 2×OCH_3 of DMT), 3.31-3.54 (m, 14H, H-1,-3,-5,-8,-10,-12 aliphatic CH_2, H-5', H-5"), 2.47-2.55 (m, 1H, H-2'), 2.15-2.21 (m, 1H, H-2"), 1.63-1.91 (m., 8H, H-2,-6,-7,-11 aliphatic CH_2).

5'-*O*-Dimethoxytrityl-4-*N*-[tris(*N,N',N''*-trifluoroacetyl)-4,9,13-triazatridecane-1-yl]-2'-deoxycytidine 3'-phosphoramidite (4). **3** (347 mg, 0.35 mmole) and tetrazole (25 mg, 0.35 mmole) were dried under reduced pressure ca 20 h. Bis(*N,N*-diisopropylamino)(2-cyanoetoxy)phosphine (105 μL, 0.35 mmole) was added to **3** previously dissolved in anhydrous dichloromethane (1 mL) under argon atmosphere. Next, tetrazole was added and dissolved gradually during the reaction. Diisopropylammonium tetrazolide precipitated and the reaction was ended after 1 h, as confirmed by the TLC analysis: R_f(B) 0.53. The solvent was evaporated under diminished pressure and the desired product was purified by silica gel chromatography ($CH_3COOC_2H_5$/TEA, 9:1) and then lyophilised from benzene to give **4** as a white solid: 340 mg, 81% yield. ^{1}H NMR ($CDCl_3$): δ (ppm) 7.79-7.88 (dd, 1H, H-6), 6.81-7.42 (m, 13H, DMT aromatic), 6.29-6.32 (m, 1H, H-1'), 5.29-5.35 (m, 1H, H-5), 4.63-4.64 (m, 1H, H-3'), 4.1-4.15 (m, 1H, H-4'), 3.79 (s, 6H, 2×OCH_3 DMT), 3.29-3.64 (m., 18H, CH_2-OP, 2×CH of i-Pr, H-1,-3,-5,-8,-10,-12 aliphatic CH_2, H-5', H-5"), 2.39-2.66 (m, 2H, CH_2-CN, H-2'), 2.21-2.25 (m, 1H, H-2"), 1.63-2.07 (m, 8H, H-2,-6,-7,-11 aliphatic CH_2), 1.03-1.28 (m, 12H, 4×CH_3 of i-Pr); ^{31}P NMR (CH_3CN): δ (ppm) 148.6, 148.9.

Hybridisation conditions

Hybridisation experiments were done in the following buffers: all containing 20 mM MOPS and 0.1 M NaCl (pH 7.2) with: 3 or 5 μM fluorescein labelled oligonucleotide d-C^{AmFl}AAAAGGGG, and 5 or 0% of formamide for more and less stringent conditions respectively. After 1 h at 37 °C all SOCL elements were removed from the hybridisation buffer, rinsed for 10 min in water and analysed under the microscope.

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