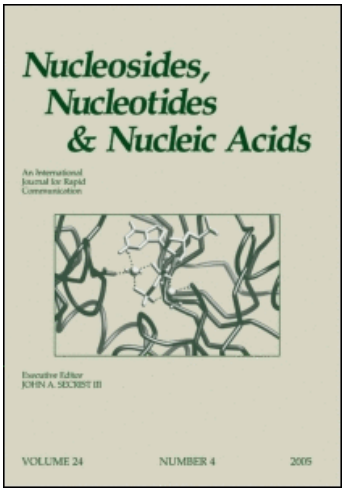

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Detection of Terminal Mismatches on DNA Duplexes in Homogeneous Assays or with Immobilized Probes

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DETECTION OF TERMINAL MISMATCHES ON DNA DUPLEXES IN HOMOGENEOUS ASSAYS OR WITH IMMOBILIZED PROBES

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□ *We recently reported the design of new fluorescent oligo-2′-deoxyribonucleotides (FODNs) for the detection of terminal mismatches on DNA duplexes in homogeneous assays. We now report the validation of this method in homogeneous assays with other sequences and the feasibility of the detection of terminal mismatches with immobilized FODNs. In all cases studied, the mismatched duplexes were more fluorescent than the perfect ones and results confirmed that the discrimination factor is sequence-dependent.*

Keywords DNA; terminal mismatch detection; fluorescent oligonucleotides; thiazole orange; non-stringent conditions

INTRODUCTION

Sequence-selective DNA analysis can be performed by hybridization of oligonucleotides (ONs) with complementary sequences.[1] However, even for short duplexes, the presence of terminal mismatches lowers the UV-melting point by only a few degrees.[2,3] For these reasons, it is difficult to find stringent conditions allowing terminal mismatch detection in a high throughput analysis with arrays of ONs in either homogeneous assays or immobilized on supports. We recently reported the design of fluorescent oligo-2′-deoxyribonucleotides (FODNs) able to discriminate between perfectly matched duplexes and those involving terminal mismatches.[4] This method is based on the use of sets of 10-mer oligo-2′-deoxyribonucleotides (ODNs) linked via their 5′-ends and varying-sized flexible polymethylene chains, to thiazole orange, with the linker being attached to the benzothiazole moiety (ODN-TO′). The mismatched duplexes were more fluorescent

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than the perfect ones (at least more than two-fold) and depended on the linker lengths used to connect the ODNs and TO′. The greatest differences were obtained with a seven methylene linker when the 5′-terminal nucleic bases on the ODN probes were purines and with a five methylene linker when they were pyrimidines. We now report the validation of this method in homogeneous assays with other sequences and the feasibility of the detection of terminal mismatches with immobilized FODNs.

RESULTS AND DISCUSSION

As models we chose to study mutations of the twelfth codon of the *ras* gene[5] as well as mutations of the CFTR gene[6] (see sequences in Table 1). The synthesis of the ODN-TO′ conjugates **[5–8, 12** and **17**] for homogeneous hybridization assays was performed as previously reported by postsynthetic reaction of a thiophosphate group incorporated at the 5′-end of the ODN probes and an halogenoalkyl function present on the polymethylene linker attached to the benzothiazole moiety of the TO′.[4] For the hybridization studies on support we chose to synthesize first the ODN-TO′ conjugates, in order to assess their integrity, and then to attach them to the support (see structure in Figure 1). The synthesis were performed at a 1 μmole scale on a modified support enabling, after the deprotection step, the recovery of ODNs ending at their 3′-ends with an aminohexyl linker.[8] Two hexaethylene glycol tethers were inserted between the aminohexyl linker and the ODN in order to avoid steric effects during the hybridization of the ODN-TO′ probes with the target sequences. After assembly of the ODNs, a masked thiophosphate group was incorporated at their 5′-ends. The deprotection of ODNs as well as their release from the support were achieved by overnight

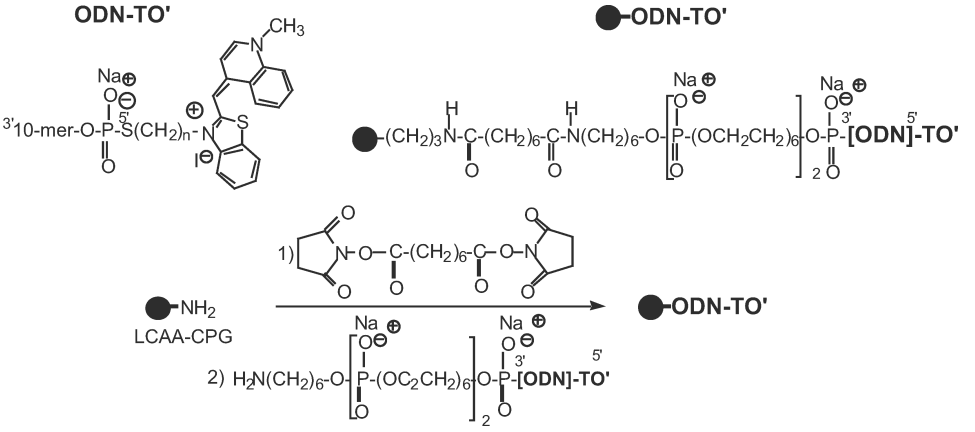

FIGURE 1 Structures of the free and immobilized ODN-TO′ conjugates.

TABLE 1 Sequences used for the detection of base mutations of the twelfth codon of *ras* gene [G into A, C, or T at the second base (targets **1–4** and ODN-TO′ probes **5–8**) and G into T, C, or A at the first base (targets **1** and **9–11** and ODN-TO′ probes **12** and **13**)] and mutations of the CFTR gene [T into G or A at position 3434 (targets **14–16** and ODN-TO′ probes **17** and **18**)]. The F_{hyb}/F_{unyb} ratios are reported (data in bold correspond to the perfectly matched duplexes) as well as the discrimination factors (italics). Errors in fluorescence values are estimated to be ±10%.

Target sequences

1 5′GTAGTTGGAGCTG**G**TG3′
2 5′TAGTTGGAGCTG**A**TGG3′
3 5′TAGTTGGAGCTG**C**TGG3′
4 5′TAGTTGGAGCTG**T**TGG3′

ODN-TO′

5 3′AACCTCGAC**C**5′ p-S $(CH_2)_5$ TO′
6 3′AACCTCGAC**T**5′ p-S $(CH_2)_5$ TO′
7 3′AACCTCGAC**G**5′ p-S $(CH_2)_7$ TO′
8 3′AACCTCGAC**A**5′ p-S $(CH_2)_7$ TO′

ODN-TO′	Target sequences 1		2		3		4	
5	**0.28**	—	1.03	*3.67*	1.09	*3.89*	1.00	*3.57*
6	3.78	*2.74*	**1.38**	—	4.22	*3.05*	3.99	*2.89*
7	2.09	*2.40*	2.48	*2.85*	**0.87**	—	*1.90*	2.18
8	3.50	*2.80*	4.86	*3.88*	4.09	*3.27*	**1.25**	—

Target sequences

1 5′GTAGTTGGAGCT**G**GTG3′
9 5′GTAGTTGGAGCT**T**GTG3′
10 5′GTAGTTGGAGCT**C**GTG3′
11 5′GTAGTTGGAGCTA**G**TG3′

Target sequences

14 5′GCTGGTTCCAAA**T**GAG3′
15 5′GCTGGTTCCAAA**G**GAG3′
16 5′GCTGGTTCCAAAAGAG3′

ODN-TO′ and **• – ODN-TO′**

12 3′CAACCTCGAA**A**5′p-S$(CH_2)_7$ TO′
13 • – 3′CAACCTCGAA**A** 5′p-S$(CH_2)_7$ TO′

ODN-TO′ and **• – ODN-TO′**

17 3′CCAAGGTTTA**A**5′p-S$(CH_2)_7$-TO′
18 • – 3′CCAAGGTTTA**A** 5′p-S$(CH_2)_7$-TO′

ODN-TO′	Target sequences 1		9		10		11	
12	1.68	*2.33*	**0.72**	—	1.55	*2.15*	1.69	*2.34*
13	0.79	*1.75*	**0.45**	—	0.95	*2.11*	0.86	*1.91*

ODN-TO′	Target sequences 14			15		16	
17[4]	**0.40**	—	1.66	*4.16*	2.50	*6.25*	
18	**0.39**	—	1.01	*2.59*	1.13	*2.89*	

treatment at 55°C with concentrated aqueous ammonia (28%) in the presence of DTT. After purification by reversed-phase chromatography, the TO′ label was coupled as above and the integrity of the new conjugates [**13** and **18**] was assessed by mass spectrometry analysis.[7] The conjugates [**13** and **18**] were then attached through their 3′-ends to a Long Chain Alkyl Amine CPG (LCAA-CPG 500) support via a two-step procedure. First, the support and the suberic acid bis-(N-hydroxysuccinimide) ester were incubated in dry DMF in the presence of DIEA overnight at room temperature. After extensive washing with DMF, CH_3OH, and CH_2Cl_2 and drying, the support was acetylated in order to prevent interactions between the unreacted amino groups of the LCAA-CPG 500 support and the phosphate groups of the conjugates and/or the target sequences. Finally, the support was incubated with solutions of the conjugates involving the aminolinkers overnight at room temperature. After extensive washing and drying, the immobilized probes **13** and **18** were used in hybridization assays.

Steady-state fluorescence experiments were performed with either 1 μM solutions (0.1 mL) of the free ODN-TO′ probes or 4–5 mg of the CPG functionalized with the corresponding ODN-TO′ probes in a 5 mM cacodylate

buffer, pH 6, containing 50 mM NaCl. Fluorescence measurements [λ_{Exc} 485 nm/λ_{Em} 530 nm] were performed with a Cytofluor II fluorescence multi-well plate reader, before and after addition of one equivalent (or more) of the target sequences to be analyzed, at either room temperature or after incubation at 6°C for different time lengths (15 minutes, 30 minutes, 45 minutes, and 1 hour 30 minutes). The results obtained at 6°C and either 30 minutes (for homogeneous assays) or 1 hour 30 (with immobilized probes) are reported in Table 1. In all cases, the F_{hyb}/F_{unhyb} ratios obtained for the perfect duplexes were lower than those obtained for the corresponding mismatched duplexes and the results confirmed the influence of the sequence on the discrimination factors.

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