Intercalating Nucleic Acids with Pyrene Nucleotide Analogues as Next-Nearest Neighbors for Excimer Fluorescence Detection of Single-Point Mutations under Nonstringent Hybridization Conditions

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Fluorescence and hybridization specificity is reported for intercalating nucleic acids (INAs), which are here oligodeoxynucleotides (ODNs) synthesized with insertions using (*S*)-1-[bis(4-methoxyphenyl)(phenyl)-methoxy]-3-[(pyren-1-yl)methoxy]propan-2-ol phosphoramidites. It is shown that an INA with two insertions placed as next-nearest neighbors can be used for discrimination between nucleic acids and their single-point mutants. Quenching of an excimer band at 480 nm is observed upon hybridizing to a complementary sequence, whereas the excimer band is present when the nucleobase pair between the two pyrene moieties is mismatched. It is the first example of a solution based on fluorescence detection of single-point mutants that uses excimer formation and does not rely on stringent hybridization conditions. Furthermore, it is shown that INAs with pyrene insertions retain their sequence specificity in thermal melting.

1. Introduction. – DNA Diagnostics is one of the fastest growing research areas, and now, with the draft of the human genome map available [1] and aiming at the full sequence in detail, the interest in the field is expected to expand even further. A map of 1.42 million single nucleotide polymorphisms (SNPs) has been described, and it has been estimated that there are 60,000 SNPs that fall within the human exons [2]. The search for sequences that differ in only one or two nucleobases creates the need for tools for detecting nucleic acid sequences that have high performance, speed, simplicity, and low cost. There have been many different techniques developed to identify mutations in nucleic acid sequences. Techniques based on matched/mismatched-duplex stabilities, restriction cleavage, ligation, nucleotide incorporation, mass spectrometry, and direct sequencing have recently been reviewed [3]. The search for new and more-efficient assay systems is still going on, and a recent example is mass-spectrometric monitoring of a PNA-based ligation reaction for multiplex detection of SNPs [4].

Pyrene is an excimer-forming molecule that has been incorporated into oligodeoxynucleotides (ODNs) by several groups. *Ebata et al*. incorporated a pyrene-modified nucleotide in the 5′-end of one ODN and a pyrene-modified nucleotide into the 3′-end of another. By hybridizing to a target sequence in a way that the pyrene moieties from the two strands come into close proximity, an excimer band at 490 nm was generated [5]. *Paris et al*. published a similar system where they also explored the utility of the

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system to detect mismatches. The ability to differentiate between a wild-type sequence (wt) and its single-point mutant (mut) is due to the ability of one of the probes to hybridize as a fully complimentary sequence to the mutant only [6]. This means that the phenomena is temperature-controlled and limits the length of the probe, and hence the selectivity, and sets high requirements for temperature control. During the last few years, there has been increasing interest in finding methods of detecting mismatches under nonstringent hybridization conditions. This requirement can be fulfilled by electro-analysis at DNA-modified electrodes [7] or by selective 2′-amine acylation [8]. A very recent method without stringent hybridization conditions is using the *MagiProbe*, which undergoes fluorescence quenching on mismatching when a quencher and a fluorophore in the probe are placed next to the mismatched base pair, whereas fluorescence is observed for perfect matching due to intercalation of the quencher [9]. This discovery has prompted us to report our findings that intercalating nucleic acids (INAs) with two pyrene intercalating moieties forming excimer complexes can be used for detection of mismatches under nonstringent hybridization conditions. The synthesis of INAs has previously been reported and is straightforward by standard oligonucleotide synthesis techniques without any post-oligo synthetic steps [10]. The INAs were synthesized by using the phosphoramidite **1** in its (2*S*)-form containing a short linkage to pyrene (*Fig. 1*). Furthermore, we wish to show that INA duplexes allow double checking of the excimer mismatching experiments by their sensitivity to mismatches on thermal melting. There are numerous examples of INAs reported in the literature, where the fluorescence properties of inserted pyrenes have been discussed [11], but there are no reports investigating how nearby mismatches in duplexes can influences the fluorescence properties of the inserted pyrenes.

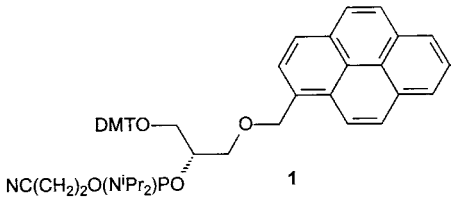

Fig. 1. *(*S*)-1-[Bis(4-methoxyphenyl)(phenyl)methoxy]-3-[(pyren-1-yl)methoxy]propan-2-ol phosphoramidite* (**1**)

2. Results and Discussion. – *Fluorescence.* As described in a previous paper, the insertion with the pure enantiomer **1** (*Fig. 1*) into an ODN forming an INA enhances the affinity for complementary single-stranded DNA (ssDNA) [10]. The stabilizing effect of the INA/DNA duplex is thought to be a consequence of intercalation of the bulging pyrene into the strands. It was shown that one nucleobase pair between two bulging insertions with **1** is sufficient to cause an increase in melting temperature of a INA/DNA duplex compared to the duplex with only one insertion. Two pyrene molecules in close proximity will, upon excitation, show an excimer band at 480 nm [12], and this is also seen when two pyrene moieties are inserted as neighbors or next-nearest neighbors in ssDNA (*Fig. 2*). The fluorescence bands at 382 and 395 nm are

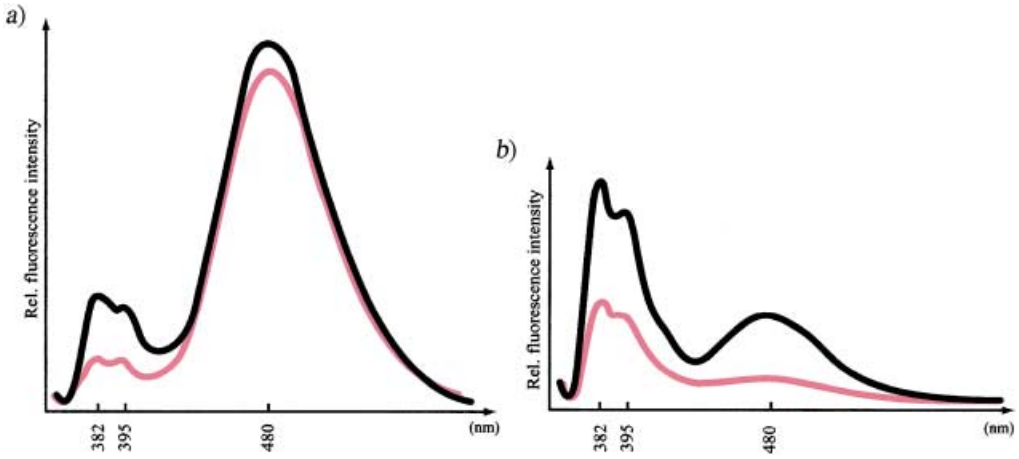

Fig. 2. *Fluorescence measurements* (P = **1**). *a*) Single-stranded INA with neighboring pyrene insertions with the sequence 5′-CTCAAGPPCAAGCT-3′ (*black*) and its duplex with the complementary strand 3′-GAGTTCGTTCGA-5′ (*red*). *b*) Single-stranded INA with next-nearest neighboring pyrene insertions with the sequence 5′-CTCAAPGPCAAGCT-3′ (*black*) and its duplex with the complementary strand 3′-GAGTTCGTTCGA-5′ (*red*).

quenched upon hybridization to the complementary strand, indicating that the bulging pyrene moiety interacts strongly with the double helix by intercalation [5][13].

On the other hand, the excimer band at 480 nm is quenched only in the duplex with one base pair between the two pyrene nucleotides (next-nearest neighbors), and not when the bulging pyrene nucleotides are neighbors (*Fig. 2*). These results imply that an intact base pair between the two pyrene nucleotides causes quenching of the excimer band. This explanation is supported by structural calculations performed with MacroModel (*Fig. 3*), where it is visualized that an intact base pair between two inserted intercalating pyrenes is possible.

The observation for the duplex bulge with two neighboring pyrenes with retained excimer fluorescence and quenching of the monomer pyrene fluorescence at 382 and 395 nm when compared with ssDNA is consistent with accommodation of the two pyrenes in intrahelical environments. In this way, stacking interactions of the pyrenes with neigboring bases can be maintained to allow quenching of the monomer fluorescence. The two bulging pyrenes are at the same time in close vicinity to each other to allow formation of an excimer complex. Examples of a similar structure of a two nucleotide bulge with formation of a stable duplex on both sides of two unpaired intrahelical bases can be found in the literature [14]. Such duplexes show bending of the duplex axis at the site of the two-base bulge. The fluorescence properties of the duplex bulge with two pyrenes differs from those observed when two neighboring nucleotides are replaced with pyrene nucleotides forming a nonbulging duplex. In the case of the pyrene monomer, fluorescence is slightly increased, and excimer fluorescence is strongly exhibited [15].

Quenching of pyrene fluorescence is a sign of strong interaction of the fluorophore with the duplex by electron transfer to or from flanking bases [16]. It was anticipated

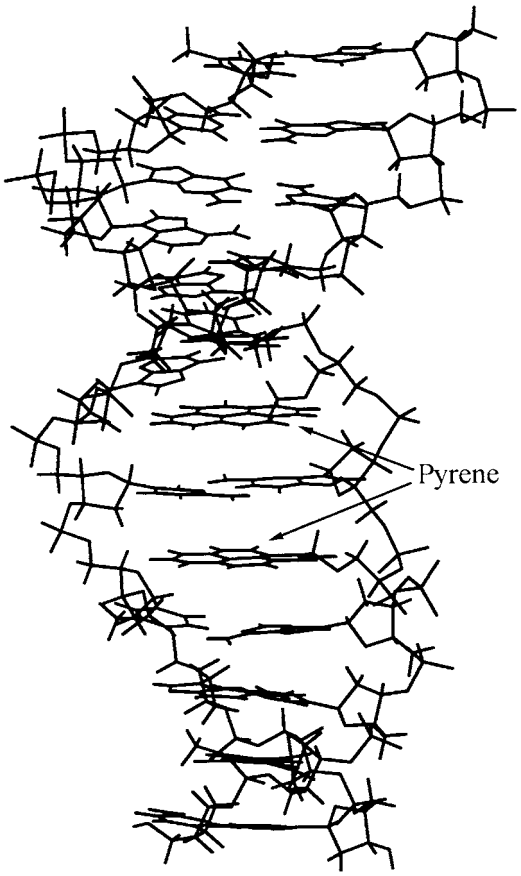

Fig. 3. *A slice of the duplex calculated in MacroModel of an INA/DNA duplex with next-nearest-neighboring pyrene insertions* (5′-CTCAPGPCAAGCT-3′ + 5′-AGCTTGCTGAG-3′, P = **1**)

that the introduction of mismatches near the site of intercalation results in increased flexibility of the pyrene and hence increased monomer fluorescence. This was also what was found irrespective of which side of the intercalator a mismatch is introduced (*Table 1*).

It was expected that a mismatch in the base pair between two pyrene moieties would not be able to quench the excimer fluorescence, and hence this could be used for the detection of single-point mutations. A probe with next-nearest-neighboring pyrene moieties was hybridized to targets with all four variants of the nucleobase (**Y**) between the two pyrene insertions, and the intensity of the excimer band at 480 nm was significantly increased (*Table 2*) when the base pair was mismatched (**Y** = G, A, T). Again it was expected that the fluorescence of the bands at 382 and 395 nm would increase upon introduction of a mismatch, which was also observed. Surprisingly, the fluorescence at 480 nm was also increased by introduction of a mismatch at the 3′-side of both intercalators (**Z** = C, A, T), indicating that the two pyrene moieties are able to

Table 1. *Fluorescence Measurements of INA with One Pyrene Insertion* (P = **1**) *Hybridized to the Complementary Sequence or to its Neighboring-Single-Point Mutants*

5′-A-G-C-T-T-**Z-Y**-T-T-G-A-G-3′
3′-T-C-G-A-A-C**P**G-A-A-C-T-C-5′

Target	**Z**	**Y**	382 nm[a])	395 nm[a])	480 nm[a])
INA alone	–	–	48	40	1
Wild type	G	C	15	12	1
Mut. 1	C	C	59	50	2
Mut. 2	A	C	75	63	2
Mut. 3	T	C	50	42	2
Mut. 4	G	T	34	29	2
Mut. 5	G	A	63	53	2

[a]) Relative intensity.

interact with each other (*Table 2*). This would only be expected if a loop large enough to let the pyrene moieties interact is created. It is noteworthy that the monomer fluorescence at 382 and 395 is quenched (compared to the unhybridized probe) when hybridized to a complementary sequence, but increased when hybridized to a sequence with one mismatch. It is, therefore, possible to use all three wavelengths (382, 295, and 480 nm) to differentiate between a fully complementary sequence and a complementary sequence with one mismatch.

Table 2. *Fluorescence Measurements of INA with Two Pyrene Insertions* (P = **1**) *Hybridized to the Complementary Sequence or to Its Single-Point Mutants*

5′-A-G-C-T-T-**Z-Y**-T-T-G-A-G-3′
3′-T-C-G-A-A-C**P**G**P**A-A-C-T-C-5′

Target	**Z**	**Y**	382 nm[a])	395 nm[a])	480 nm[a])
INA alone	–	–	44	38	17
Wild type	G	C	19	17	4
Mut. 1	C	C	84	73	14
Mut. 2	A	C	74	64	10
Mut. 3	T	C	84	74	12.5
Mut. 4	G	T	62	54	8
Mut. 5	G	G	84	74	17
Mut. 6	G	A	70	60	12

[a]) Relative intensity.

Work is now in progress on constructing DNA chips with INAs as new SNP assays. It is believed that the reliability in detecting SNPs can be improved when monomeric and excimer fluorescence properties of matched/mismatched duplexes are used in combination with conventional techniques based on matched/mismatched duplex stabilities. When the latter methods are used alone, cross-hybridization 'side reactions' may occur with other similar targets, which would reduce their reliability as diagnostic tools [17].

Thermal Denaturation Studies. Insertions of intercalators into DNA have previously been accompanied by a decrease in specificity for hybridization to fully complementary sequences when compared to sequences with mismatches in the base pairs surrounding the intercalator [18]. Experiments were aimed at testing whether this is also the case for duplexes with pyrene insertions, when mismatches are inserted to either side of the intercalator, or next to or between two intercalators. The specificity is measured by the difference in the melting temperature between the fully complementary duplex and the duplex where one mismatch has been introduced. Melting-temperature data are shown in *Table 3*.

Table 3. *Melting-Temperature Data of INAs with Different Insertion Patterns When Hybridized to the Complementary Structure or Point Mutations at Nucleotides #6 and #7* (P = **1**)

Target: 5′-A-G-C-T-T-**Z-Y**-T-T-G-A-G-3′
Probes:
I 3′-T-C-G-A-A-C-G-A-A-C-T-C-5′
II 3′-T-C-G-A-A-C**X**G-A-A-C-T-C-5′
III 3′-T-C-G-A-A-C**X**G**X**A-A-C-T-C-5′
IV 3′-T-C-G-A-A-C**X**G-A-**X**-C-T-C-5′
V 3′-T-C-G-A**X**A-C-G-A**X**A-C-T-C-5′

Target	**Z**	**Y**	**I**		**II**		**III**		**IV**		**V**	
			T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m
Wild type	G	C	47.4		50.4		51.4		45.4		60.8	
Mut. 1	C	C	23.4	– 24.0	34.0	– 16.4	38.0	– 13.4	23.4	– 22.0	33.8	– 27.0
Mut. 2	A	C	30.8	– 16.6	34.2	– 16.2	36.6	– 14.8	ND[a])		ND[a])	
Mut. 3	T	C	28.6	– 18.8	33.6	– 16.8	35.2	– 16.2	25.4	– 20.0	37.4	– 23.4
Mut. 4	G	T	36.2	– 11.2	42.2	– 8.2	45.2	– 6.2	36.6	– 8.8	45.8	– 15.0
Mut. 5	G	G	40.0	– 7.4	42.4	– 8.0	38.6	– 12.8	39.4	– 6.0	53.2	– 7.6
Mut. 6	G	A	39.8	– 7.6	39.0	– 11.4	39.0	– 12.4	39.2	– 6.2	49.0	– 11.8

[a]) ND: not determined.

The specificity against mismatches of the INA probe is in the range of that of the unmodified ODN probe, though there is a drop in selectivity for C–C mismatches to the 5′-end of the intercalation site (*Table 3*; Mut. 1 with Probe **II** and **III**). A considerable drop in selectivity is also observed in one case where the base mismatch T–G occurs in the middle of two pyrene moieties of probe **III**. A consistent trend is the higher specificity for the doubly modified probe with two intercalators separated by four nucleotides (probe **V**). When the mismatch is located in the middle of the sequence between the two intercalating pyrenes (*Table 3*), this INA in all the investigated cases is more sensitive to mismatches than the corresponding wild type ODN with the same length. Another important property of the very same INA is that it also forms a considerably stronger duplex with the matching oligo, as measured by thermal melting. We assume that these two properties in co-operation can be used for designing cheap, selective, and short oligo probes for single mismatch detection. The increase of the thermal melting temperatures of the INA/DNA duplexes when compared to the unmodified duplexes is believed to be an important factor to achieve short oligos, which are sensitive to mismatches. A similar approach has been described for locked nucleic acids (LNA), where stronger duplex formation enabled shorter sequences to be used [19][20].

3. Conclusions. – It is shown that an excimer band at 480 nm is generated when two pyrene nucleotides are inserted as neighbors or as next-nearest neighbors in the INA. Only in the latter case, with next-nearest neighbors, is quenching of the excimer band observed upon hybridization of the INA to a fully complementary strand, whereas there is no or little quenching of the excimer band if there is a mismatch between the two pyrene moieties or at the neighboring nucleobases to the 3′-side of the intercalators. The fluorescence bands at 382 and 395 nm are also partly quenched when the INA is hybridized to a fully complementary ODN, while enhanced when hybridized to a single-point mutated nucleotide. These findings can be used to discriminate between single-point mutations and wild-type ODNs without relying on differences in melting temperatures, although the latter is still possible, as it is shown that the INAs can be designed to have higher specificity combined with higher melting temperatures than the unmodified ODNs when they are hybridized to ODNs.

In short, it is shown that INAs have a potential of being used both under stringent and nonstringent hybridization conditions to differentiate between single-point mutations.

Experimental Part

1. *ODN and INA Synthesis, and Purification and Measurement of Melting Temperatures.* The ODN and INA synthesis was carried out on a *Pharmacia LKB Gene Assembler®* with Gene Assembler Special software v. 1.53. The pyrene amidite **1** [10] was dissolved in a 1:1 mixture of dry MeCN and dry CH_2Cl_2, as a 0.1M soln., and inserted into the growing oligonucleotide chain under the same conditions as for normal nucleotide couplings (2-min coupling). The coupling efficiency of the modified nucleotide was >99%. The ODNs and INAs were synthesized with DMT on and purified on a *Waters Delta Prep 3000* HPLC with a *Waters 600E* controller and a *Waters 484* detector on a *Hamilton PRP-1* column. Buffer *A:* 950 ml of 0.1M NH_4HCO_3 and 50 ml of MeCN (pH 9.0); buffer *B:* 250 ml of 0.1M NH_4HCO_3 and 750 ml of MeCN (pH 9.0). Gradients: 5 min 100% *A*, linear gradient to 100% *B* in 40 min, 5 min with 100% *B*, linear gradient to 100% *A* in 1 min and then 100% *A* in 29 min (product peak at *ca.* 37 min). The ODNs and INAs were DMT deprotected in 925 µl of H_2O and 75 µl of AcOH, and purified by HPLC, again with the same column, buffer system, and gradients (product peak at *ca.* 26 min). To eluminate the salts, the ODNs and INAs were redissolved in 1 ml of H_2O and concentrated *in vacuo* three times.

All ODNs and INAs were confirmed by MALDI-TOF analysis on a *Voyager Elite Biospectrometry Research Station* from *PerSeptive Biosystems*. The transition-state analyses were carried out on a *Perkin-Elmer* UV/VIS spectrometer *Lambda 2* with a *PTP-6* temp. programmer by using PETEMP rev. 5.1 software and PECSS software package v. 4.3. All ODNs were measured in 120 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA (pH 7.0, 3.0 µM each strand). All melting temp. are with an uncertainty $\pm 0.5°$ as determined by repetitive experiments.

2. *Molecular Calculations.* The molecular calculations used the Amber force field performed in MacroModel 7.0 with H_2O as solvent, and minimization was done by the conjugant gradient method. The starting oligonucleotide sequence for calculation with the inserted pyrenes was taken from *Brookhavens Protein Databank*, and modified in MacroModel before minimization. The sequence is a modified 13-mer highly conserved HIV-1 long terminal repeat region [21]. A-5 and G-7 were replaced by pyrene nucleotide 1, and the opposite nucleotides were deleted. The pyrenes were placed in the interior of the duplex from the beginning. All bonds were free to move and to rotate.

3. *Fluorescence Measurements.* Fluorescence measurements were carried out with a *Perkin-Elmer MPF-3* instrument in 2-ml plastic cuvettes with excitation at 340 nm and detection at 360–600 nm. All measurements were conducted in 150 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA (pH 7.0), with a concentration of 1.5 µM of each strand. The plastic cuvettes did not interfere with the measurements.

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