Probing DNA sequences in solution with a monomer–excimer fluorescence color change

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ABSTRACT

The use of a simple fluorescent nucleoside analogue in detection of point mutations by hybridization in solution is described. Pyrene is placed at 3′ and 5′ ends of a pair of oligodeoxynucleotide probes via a phosphoramidite derivative of deoxyribose with this fluorophore attached at the 1′ position, replacing a DNA base. Adjacent binding of dual probes containing this fluorophore to a complementary target sequence results in a pronounced spectral change from blue pyrene monomer emission (λ\text{max} = 381 398 nm) to green–white excimer emission (λ\text{max} = 490 nm). Optimization of the relative binding positions of the two probes shows that the greatest spectral change occurs when they bind with partial end overlap. In optimum orientation, the monomer emission band for the probes decreases intensity by as much as a factor of seven and the excimer band increases up to 40-fold on binding a complementary target. Application to the detection of a single-base point mutation in solution is described.

INTRODUCTION

The rapidly increasing pace of DNA sequencing in recent years has begun to make available much information on the underlying genetic causes of inherited and acquired diseases. It is becoming clear that in the future, methods for rapidly screening patient-derived samples for known disease-related sequences will be an important tool in the arsenal for prevention and treatment of disease. Of the many techniques which are being developed for DNA sequence identification, solution-based fluorescence hybridization methods offer a number of advantages which make them the tool of choice for engendering a change in spectral properties when the DNA target of interest is detected, relative to other sequences which may be present in the sample. For example, useful changes can result from quenching or loss of quenching of fluorescence which is triggered by hybridization. Noteworthy in this respect is the work of Kramer et al. (7,13), who have used hairpin-forming oligonucleotides which carry a fluorescence-quenching species on one end and a fluorescent dye on the other. On hybridizing to a target, a considerable increase in fluorescence emission intensity is observed. Changes in fluorescence intensity on hybridization have been noted in other solution-based strategies as well.

It is often advantageous in solution-based sequence probing to utilize a change in fluorescence emission wavelength, rather than in intensity alone. This can result in a more definitive answer as to the presence or absence of a specific sequence in the target DNA, and ratios of intensities at two wavelengths can be used to define the answer and avoid interference from background fluorescence of unbound probes and other fluorescent species in a given sample. One of the most useful color-changing strategies is the use of fluorescence resonance energy transfer (FRET) between a donor and acceptor dye (1). For example, FRET can be used to detect single base pair changes in a template-directed primer extension reaction (10) or specific sequences being amplified in the PCR reaction (9). In a related finding, it has been shown that luminescence resonance energy transfer (LRET) can also occur between sensitized lanthanide metals and acceptor dyes (6), an effect which can be used to distinguish mismatched DNA targets from fully complementary targets (5).

A third example of a two-chromophore system which gives a color change is the use of excimer-forming dyes. Pyrene is a well-characterized excimer-forming fluorophore which has been incorporated into synthetic oligonucleotides by several research groups (2,4,11,12,15–26). A number of these have observed excimer fluorescence (2,4,11,12,21). Two recent studies have made use of excimer formation by adjacent pyrenes as a color-changing reporter of the presence of specific sequences in solution. Ebata et al. (4) used singly pyrene labeled oligomers where the presence of excimer emission indicated hybridization. The utility of that system for mismatch detection was not explored. Lewis et al. (11) used a probe with a bispyrenyl fluorophore at the 5′ terminus to distinguish between a target with a mismatch and its Watson–Crick complement. In that case the probe alone displayed excimer emission, and the excimer intensity increased when the probe hybridized to a complementary target.

We recently reported a new and simple chemical strategy for the incorporation of single or multiple pyrene fluorophores into DNA oligonucleotides (26,27). This approach makes use of a pyrene derivative in which the fluorophore replaces the DNA base of a standard deoxyribonucleoside (Fig. 1). This compound is utilized as the phosphoramidite derivative, which allows for automated incorporation into a sequence at any position during

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DNA synthesis rather than post-synthesis labeling. Moreover, because of the placement of the fluorophore in a position analogous to a DNA base, it is well situated to form excimers with other adjacent pyrene residues. This led us to hypothesize that adjacent hybridization of two short DNA oligomers carrying pyrene nucleosides might result in significant amounts of excimer emission, engendering a spectral change for a specific target sequence.

We now report studies in which we explore the use of this new fluorescent nucleoside analogue in dual oligonucleotide probes. We find that with optimal spacing of the hybridizing probes, adjacent binding results in the appearance of efficient excimer emission, coupled with a large drop in monomer emission. The changes in excimer and monomer intensity are expected to result in location of the pyrene end labels directly adjacent to one another. To investigate the effects of relative positions of hybridization, the sequence context utilized for initial probe design was the H-ras oncogene containing a well-characterized G→T transversion in codon 12 (30). A pair of singly labeled pyrene-containing 10 nt oligomers was synthesized and designed to bind to a portion of the gene including codon 12. The sequences were d(GGCGCCGCTTGGCCGCG) (P1) and d(GGCCTGGGACGGCGCG) (P2), where P in each sequence represents the pyrene nucleoside. The sequences are predicted to allow contiguous hybridization of the two probes when hybridized as predicted (Fig. 2), where for N=1, N+0, and N+1; these designators give the relative spacing of the two probes when hybridized as predicted (Fig. 2), where for

**RESULTS**

**Sequence design**

Since pyrene excimer formation depends strongly on a well-defined geometry of fluorophore interactions (29), experiments were aimed at investigating not only effects of single and dual probe hybridization, but also the effects of relative positions of hybridization. The sequence context utilized for initial probe testing was the H-ras oncogene containing a well-characterized G→T transversion in codon 12 (30). A pair of singly labeled pyrene-containing 10 nt oligomers was synthesized and designed to bind to a portion of the gene including codon 12. The sequences were d(GGCGCCGCTTGGCCGCG) (P1) and d(GGCCTGGGACGGCGCG) (P2), where P in each sequence represents the pyrene nucleoside. The sequences are predicted to allow contiguous binding on the H-ras target sequence. A target oligomer 40 nt in length corresponding to the mutant ras sequence was also synthesized (Fig. 1); it is denoted (N+0).

Hybridization of the P1 and P2 probes to the N+0 target is expected to result in location of the pyrene end labels directly adjacent to one another. To investigate the effects of relative pyrene orientation on spectral changes, we synthesized a series of additional targets (derived from N+0) in which bases were deleted or added sequentially to decrease or increase the formal distance between the pyrene-labeled probe ends (Fig. 2). An alternative strategy would have been to delete or add bases next to pyrene in the probes themselves; however, the former strategy was chosen instead to avoid the well-known differences in quenching of pyrene fluorescence with varying neighboring bases (31).

**Oligodeoxyribonucleotide synthesis**

DNA oligonucleotides were synthesized on an Applied Biosystems 392 synthesizer using standard β-cyanoethylphosphoramidite chemistry but with extended (12.5 min) coupling cycles for the pyrene residues, as described (26,27). A pyrene phosphoramidite concentration of 0.05 M in acetonitrile was used, and shown not to compromise coupling efficiency. Universal CPG columns (ClonTech) were utilized for introduction of the label at the 3′ end of probes. DNA oligomers were purified by preparative 20% denaturing polyacrylamide gel electrophoresis and isolated by the crush and soak method. Molar extinction coefficients of unmodified oligonucleotides were calculated by the nearest neighbor method (28). Concentrations of oligonucleotides containing pyrene residues were determined in the following way: UV absorbances were measured at 260 and 350 nm. The 260 nm values were substituted into Beer’s Law, using the calculated extinction coefficient for the DNA portion of the oligonucleotide. A correction factor for the contribution of pyrene at 260 nm was taken to be 0.5 × the absorbance at 350 nm.

**Steady-state fluorescence measurements**

The steady-state fluorescence spectra were recorded on a SPEX-Fluorolog-2 series fluorometer at room temperature. The source of radiation was a xenon arc lamp. An excitation wavelength of 350 nm was used, and all excitation and emission slits were set to 2 mm, resulting in ~3.4 nm resolution. Fluorescence measurements were taken in the right angle mode. Each oligomer was present at a concentration of 0.4 mM in a pH 7.0 buffer (10 mM Na·Pipes, 10 mM MgCl₂, 100 mM NaCl). The buffer solutions were air-saturated. For the duplex measurements, a 15 min hybridization time ensued after adding and mixing in the target strand. All emission spectra were measured using a reference dye (rhodamine-B) to compensate for lamp fluctuations, and were corrected for instrument response as well as by subtraction of data for buffer alone.

**MATERIALS AND METHODS**

**Synthesis of pyrene-1′-α-deoxynucleoside-5′-dimethoxytrityl-3′-phosphoramidite**

The phosphoramidite derivative of this pyrene nucleoside was prepared as previously described (27). The 1H-NMR spectrum was identical to the reported one.

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In all, five different target DNAs were synthesized to test spacing effects. This allowed testing of five different spatial orientations in a systematic way. They are designated N–3, N–2, N–1, N+0 and N+1; these designators give the relative spacing of the two probes when hybridized as predicted (Fig. 2), where, for
Figure 2. Illustration of the relative spacing of dual pyrene probes P1 and P2 on the series of target DNAs designated N+1, N+0, N–1, N–2, N–3. Note that this relative spacing is formal (based on sequence only) and does not necessarily correspond to actual interchromophore distances.

Example, N–3 indicates three formal nucleotides overlapping on hybridization (pyrene is counted as a nucleotide) and N+0 indicates directly adjacent binding with pyrenes expected to be side-by-side. The N+1 sequence should separate the pyrene labeled ends formally by 1 nt of intervening distance.

Effects of hybridization of single and dual probes

Fluorescence measurements were made for solutions of probes P1 and P2 in the absence of target as well as individually bound and tandemly bound to the complementary targets. Figure 3 shows the results for probes P1 and P2 with the N–1 target. On excitation at 350 nm, individual probes showed fluorescence typical of pyrene monomer emission (λ<sub>max</sub> = 381–398 nm) (32). Addition of target strands to single probes resulted in quenching of monomer emission in all cases, by a factor of ~3–5, which is probably due to quenching by nearby DNA bases (31). However, addition of targets to both probes together resulted in a simultaneous drop in the monomer emission and appearance of a new broad emission band (λ<sub>max</sub> = 490 nm) characteristic of pyrene excimer fluorescence (32). This effect was seen for all the targets studied. For example, with the N–1 target, the monomer emission at 398 nm dropped to less than one-third of its original value, while the increase in emission intensity at 490 nm was nearly 2-fold (Fig. 3).

Effect of spacing on spectral change

The two probes P1 and P2 were then hybridized to the five targets N–3 through N+1 and the effects on fluorescence were measured. The spectra are shown in Figure 4. Interestingly, the N+0 spacing does not result in the largest excimer:monomer ratio (as measured by intensities at 490 and 398 nm), despite the close proximity of pyrenes expected for that case. The most favorable arrangement for maximum excimer fluorescence upon hybridization of neighboring probes was seen with the N–2 target. A 2:0:1 excimer:monomer ratio resulted from this arrangement of probes, corresponding to a 5-fold decrease in monomer emission upon hybridization along with a simultaneous 3-fold increase in excimer emission. Single-base changes in spacing from this optimum, corresponding to a single-base deletion or insertion in a target, resulted in significant drops in excimer:monomer ratio, with the N–1 case giving a 0:6:1 ratio, and the N–3 case a 1:2:1 ratio. The greatest probe separation examined (with the N+1 target) showed much less emission at the wavelength corresponding to excimer, with a 490:398 nm emission ratio of 0.25:1.
Optimized design for ras codon 12 point mutation

Once optimized spacing of the tandem probes was established, studies were aimed at testing the utility of dual pyrene probes for point mutation detection in the H-ras oncogene. Two new probes, 7 and 13 nt in length (designated P3 and P4), were synthesized such that codon 12 was centered on the shorter probe. The sequences are d(CCGTCGGP) and d(PTGTGGGCAAGAGT), respectively, which should result in hybridization with the N–2 spacing. Two new targets were synthesized, 50 nt in length, corresponding to the authentic protooncogene (WT) and the oncogene with the known codon 12 mutation (MT). These strands are complements of the initially studied target sequence. With these sequences, probe P4 can bind both WT and MT DNAs; however P3 is only completely complementary to MT which possesses the C→A point mutation. The WT target possesses the unmutated sequence, thereby presenting a C-T mismatch for probe P3, which is expected to decrease its affinity substantially.

Effect of point mutation on signal

Fluorescence spectra were then measured for the combined P3 and P4 probes in the absence or presence of targets. Once again, almost no emission at excimer wavelengths (490 nm) is observed without targets (Fig. 5); the 490/398 nm intensity ratio is 0.01. The only case in which significant excimer emission was observed was when P3 and P4 were both in the presence of the mutant target (MT) which presents no base mismatches for either probe. The relative increase in intensity of the 490 nm band on binding of probes to the MT target was 40-fold, which was considerably greater than the above N–2 case, due to the lower 490 nm emission intensity of the unbound P3 and P4 probes. A 7-fold decrease in intensity at 398 nm was observed simultaneous with the increase in the excimer band intensity. The resulting excimer:monomer ratio was 2.7:1, similar to the N–2 case described above. By contrast, addition of the unmutated target (WT) to the same probes resulted in a slight decrease in 490 nm emission as well as a 4.7-fold decrease in 398 nm monomer emission; the resulting excimer:monomer ratio was 0.04, very different from that for the sequence with a single-base point mutation.

We then tested whether the presence of an equal amount of WT target would affect the ability of P3 and P4 to bind and report on the MT sequence. As in the case with MT target alone, excimer emission and a drop in monomer emission also resulted when both mutant and normal targets were presented, simultaneously in a 1:1 ratio, to a solution of free P3 and P4 probes (data not shown). Thus, the data show that the presence of a target that has a one base change does not obscure the excimer signal.

Visual examination of solutions containing probes alone as well as solutions after addition of target strands shows that this spectral change is clearly distinguishable to the naked eye (Fig. 6). The probes alone in buffer appear faint blue in color; addition of the complementary target strand produces a white fluorescence with a marked increase in apparent intensity. However, addition of the singly mismatched 50mer target produces a smaller effect in the opposite direction, i.e. the weak blue fluorescence appears even more faint than the case with unbound probes alone.

To test the limits of detection and sequence discrimination by probes P3 and P4, we carried out a series of dilutions to give from 400 nM to 400 pM DNA concentration. We examined the probes alone as well as in the presence of equimolar amounts of targets WT and MT; the data are summarized in Figure 7. Wavelengths at the short-wavelength peak of monomer (381 nm) and long-wavelength shoulder of excimer (510 nm) were used to calculate emission intensity ratios, in order to lessen spectral overlap. The results show that the complementary target (MT) can be detected to as low as 1.3–4 nM. The probes also discriminate the singly mismatched target (WT) from the complementary one at concentrations as low as 4–13 nM. At the lowest concentrations the instrument response was limiting.

DISCUSSION

The results show that optimum spacing of adjacent hybridized pyrene labeled oligomers occurs with the N–2 arrangement, with formally 2 nt of overlap. It is not clear what the structural origin of this is, although presumably the two fluorophores are located such that pyrene–pyrene interactions are more favorable for reaching the excimer geometry. The result is a 5-fold drop in monomer emission (398 nm) and a 3-fold increase in excimer emission (489 nm) on binding. This gives an easily detectable change when monitored by spectrometer or even by visual inspection. A 2.7:1 excimer to monomer ratio exceeds the 1:1 ratio previously reported for a related approach utilizing pyrenes...
attached post-synthetically by longer tethers (4). We surmise that the more rigid fluorophore attachment in the present case would favor interchromophore interaction with less entropic cost; however, structural information is needed before conclusions can be drawn in this regard. A 2:1 excimer:monomer ratio was reported for binding of a probe containing two pyrenes internally located (12); however, excimer fluorescence was also seen for the unbound probe as well, thus giving a smaller change than in the present case.

Previous work has shown that pyrene labels can be used to generate excimer signals in adjacently bound probes (4). The present results show that more pronounced spectral changes accompany the use of our pyrene label, in which the fluorophore is directly attached to the DNA backbone. We have also demonstrated that the present dual probes are effective in detection of point mutations, which was not explored in the previous study. In addition, our spacing studies have shown an optimum spectral change at the N–2 spacing; in the previous work spacing was not decreased further than the case corresponding to N+0, and so it remains to be seen whether a greater change might be seen for those probes in a different relative placement. Another advantage of the current approach is the ease of synthesis of the fluorophore and its ready incorporation into DNA strands during standard automated synthesis. The earlier study (4) utilized two different pyrene fluorophores which are more complex, and which require post-synthesis labeling of the DNA.

Dual pyrene-labeled probes such as the P3/P4 pair are likely to be useful in detection and identification of sequence translocations, and have been shown here to be highly responsive even to a single-base point mutation. In addition, it is notable that significant changes in excimer:monomer ratio were also seen for single-base insertions and deletions, a fact which might broaden the application of such a dual probe strategy. Of course, the generality of the approach for sequences other than the present H-ras targets remains to be demonstrated.

Our pyrene dual probe strategy is particularly simple to carry out and interpret for several reasons. First, it does not require specialized instrumentation beyond a simple spectrophotometer, and does not require time-resolved measurements. Since there is excimer emission only when the probes are bound to a fully complementary target, background fluorescence does not present a problem such as may be the case with strategies that utilize two pyrenes in a single probe (2,11,12,25). In addition, the large Stokes shift of pyrene excimer fluorescence (140 nm) allows the avoidance of spectral overlap and interference that can occur with FRET-based strategies. Finally, the present labeling method is quite simple since it utilizes a phosphoramidite derivative that can be incorporated at any position in a sequence during DNA synthesis.

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