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# Conformation and dynamics of nucleotides in bulges and symmetric internal loops in duplex DNA studied by EPR and fluorescence spectroscopies

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ABSTRACT

The dynamics and conformation of base bulges and internal loops in duplex DNA were studied using the bifunctional spectroscopic probe **Ç**, which becomes fluorescent (**Ç$^{f}$**) upon reduction of the nitroxide functional group, along with EPR and fluorescence spectroscopies. A one-base bulge was in a conformational equilibrium between looped-out and stacked states, the former favored at higher temperature and the latter at lower temperature. Stacking of bulge bases was favored in two- and three-base bulges, independent of temperature, resulting in DNA bending as evidenced by increased fluorescence of **Ç$^{f}$**. EPR spectra of **Ç**-labeled three-, four- and five-base symmetrical interior DNA bulges at 20 °C showed low mobility, indicating that the spin-label was stacked within the loop. The spin-label mobility at 37 °C increased as the loops became larger. A considerable variation in fluorescence between different loops was observed, as well as a temperature-dependence within constructs. Fluorescence unexpectedly increased as the size of the loop decreased at 2 °C. Fluorescence of the smallest loops, where a single T·T mismatch was located between the stem region and the probe, was even larger than for the single strand, indicating a considerable local structural deformation of these loops from regular B-DNA. These results show the value of combining EPR and fluorescence spectroscopy to study non-helical regions of nucleic acids.



## 1. Introduction

Nucleic acids fold into different secondary and tertiary structures to enable various biological functions, with bulges and loops being abundant. Bulges are formed when one strand within a duplex is longer than the other strand, i.e. when there are nucleotides in a duplex region that are not involved in canonical base-pairing. Loops can be at the end of a duplex region (hairpin loops) or within duplexes containing consecutive mismatches (e.g. T·T, U·U or G·A, internal or interior loops). Internal loops can be either symmetric (both strands in the loop are of the same length) or asymmetric.

Bulges and loops can have different effects on DNA structure. They can distort the stacking of bases in the duplex [1,2], induce a bend in the nucleic acid [3,4], reduce the stability of the helix [5], and/or increase the major groove accessibility at base-pairs flanking the bulge [6]. Bulges are often intermediates for errors in DNA replication, targets for repair enzymes in imperfect homologous recombination [7], and are believed to play a significant role in many diseases, including muscular dystrophy and Alzheimer's disease [8]. Single nucleotide polymorphisms that account for ~90% of mutations in an individual's DNA also include some forms of single-base bulges [9]. RNA contains an abundance of internal loops that are important for their function and often targets for drugs, for example the bacterial ribosome [10] and the viral TAR-RNA [11]. Understanding the structure and stability of bulges, loops and mismatches is also essential for prediction of mishybridization events in all nucleic acid hybridization assays such as northern blots, RT-PCR and *in situ* hybridization assays.

Bulged nucleic acids have been studied by different biophysical techniques. NMR studies of single nucleotide bulges have shown that bulged purines prefer to stack within the helix while pyrimidines either loop out or stack, depending on the identity of the base, flanking sequence and temperature [1,12,13]. Fluorescence spectroscopy, utilizing fluorescent nucleoside analogs to elucidate structural perturbations within nucleic acids [14], has been used to study the structure and dynamics of bulges [15,16], hairpins [17,18] and loops [15,19]. Furthermore, gel electrophoretic mobility, FRET measurements and electron microscopy have showed that bulges introduce kinks into DNA helices [20].

X-ray crystallography has infrequently been used to study DNA bulges or loops. In one example, crystallographic analysis showed a looped-out structure of a single-nucleotide adenine (A) bulge, while an NMR study of the same sequence showed that the A stacked into the helix [21]. These apparently conflicting results show the importance of using more than one technique for studying conformations of non-duplex nucleic acid structures. Furthermore, it is important to obtain information about conformational dynamics.

Electron paramagnetic resonance (EPR) spectroscopy is a useful technique for studying both structure and dynamics, but has not

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been frequently applied to bulges and loops. EPR studies of nucleic acids require site-directed spin-labeling [22]. We have used EPR to study the mobility of the bulge in the TAR-RNA after incorporation of a probe into the 2′-position of selected nucleotides [23]. Information about TAR-dynamics gave insight into structural changes upon binding to a variety of different ligands, such as metal ions [24], small organic molecules [25] and peptides [26].

The rigid nitroxide nucleoside **Ç** [27], which forms a stable base-pair with guanine (Fig. 1) [28], reports directly on the motion of the base to which the nitroxide is fused, unlike most other labels that are connected with a flexible tether. **Ç** has been used to investigate the dynamics of DNA hairpin loops [29], bulges [30], folding of the DNA cocaine aptamer [31] and measure long-range distances and orientations [32,33]. It has also enabled the study of internal motions of DNA duplexes by EPR using either continuous wave- [34] or pulsed-EPR spectroscopy [35]. The nitroxide functional group can be reduced to the corresponding amine, which is strongly fluorescent [36]. Thus, the two almost identical labels, **Ç** and **Ç$^f$** (Fig. 1), can be used to study the same sample with two spectroscopic techniques. In a previous study of conformation and dynamics of nucleotides in hairpin loops, we found the fluorescence data to be fully consistent with the EPR results, where higher quantum yield corresponded to higher mobility of the labeled nucleotide [29]. However, there have also been cases where fluorescence data from **Ç$^f$** have complemented the EPR results, by giving additional insights into conformational equilibria. For example, unusually large changes in fluorescence of a base-paired nucleotide at a helical junction, which had limited mobility as shown by EPR, indicated a helical tilt during folding of the cocaine aptamer [31].

We have previously used EPR spectroscopy to study the conformational dynamics of bulges in duplex DNA, including a single-bulged nucleotide that was able to participate in base-pair exchange with nucleotides in an adjacent mismatch [30]. In this paper, we extend the study of simple one- to three-base bulges to include fluorescence spectroscopy. Both the EPR and fluorescence data indicated that the single-base bulge was in a temperature-dependent equilibrium between a stacked and a looped-out conformation while bases in two- and three-base bulges were stacked into the helix. The EPR studies of less studied symmetrical internal loops demonstrated direct correlation between size of the loops and their mobility at 37 °C. Unexpectedly high fluorescence of the smallest and most immobile loops indicated local structural perturbations.

## 2. Materials and methods

### 2.1. General

Water was purified on a MILLI-Q water purification system. DNA oligomers were synthesized on an ASM 800 DNA synthesizer from Biosset (Russia). All commercial phosphoramidites and columns were purchased from ChemGenes. Solvents and reagents were purchased from ChemGenes, Sigma–Aldrich and Applied Biosystems. Molecular weight (MW) of DNA was determined by MALDI-Tof analysis and mass spectra recorded on a Bruker Autoflex III. UV–vis spectra were recorded on a PerkinElmer Lambda 25 UV–vis spectrometer. Continuous wave (CW) EPR spectra were recorded on a MiniScope MS200 (Magnettech, Germany) X-band spectrometer. Steady-state fluorescence measurements were carried out in a macro fluorescence cell (Spectrocell, USA) with a path length of 0.5 cm on a SPEX FluoroMax spectrometer.

### 2.2. DNA synthesis and purification

The probe was site-specifically incorporated into DNA by manual coupling, purified and quantified as described before [29]. Spin-labeled DNA was reduced with $Na_2S$, purified by gel electrophoresis [36] and characterized by MALDI-Tof [29].

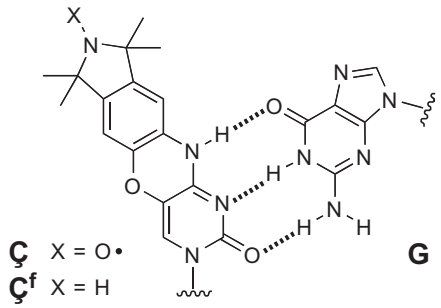


**Fig. 1.** Rigid spin-label **Ç** and reduced spin-label **Ç$^f$** base-paired to guanine.

### 2.3. Spectroscopic measurements

EPR spectra of spin-labeled DNA duplexes (final conc. 200 μM of duplex) were prepared in PNE buffer (10 mM $Na_2HPO_4$, 100 mM NaCl, 0.1 mM $Na_2EDTA$, pH 7.0; 10 μL), the samples were placed in a quartz capillary and EPR spectra collected as previously described [29]. All fluorescent DNA samples were measured at 12.5 μM in 400 μL of PNE buffer using an excitation wavelength of 365.5 nm [36]. Fluorescence spectra were averaged over five scans. Quantum yields were determined as previously described [36].

## 3. Results and discussion

### 3.1. Bulges containing one- to three-bases

For preparation of DNA duplexes containing bulged nucleotides, the spin-labeled DNA 14-mer (**x**) (Fig. 2) was synthesized and annealed to shorter complementary strands to form one- (**X2**), two- (**X3**) or three-base (**X4**) bulges, respectively (Fig. 2). The X-band CW EPR data was subsequently collected at 2 and 20 °C (Fig. 2).

The EPR spectrum of the one-base bulge (**X2**) at 20 °C contains a fast-motion component that has similar mobility as the single-stranded DNA (**x**). Thus, the EPR spectrum reflects a mixture of single-stranded-like spectrum and a duplex-like spectrum, indicating that the bulged **Ç** can be present in either a looped-out or a stacked conformation [30], consistent with early optical and photochemical experiments on synthetic RNAs showing that extra pyrimidines can loop out [37,38]. Furthermore, NMR analysis of a 13-mer DNA containing a thymidine (T) in a one-base bulge has previously shown that the extra T is in a conformational equilibrium between looped-out and stacked states [1]. However, when the temperature of **X2** was lowered to 2 °C, the EPR spectrum became similar to all others (Fig. 2), indicating that stacking of **Ç** is favored at low temperatures.

The EPR spectra of the two- (**X3**), and three-base (**X4**) bulges at both temperatures are similar to the EPR spectrum of the fully base-paired duplex (**X1**) (Fig. 2). The reduced mobility relative to the one-base bulge is consistent with stacking. NMR studies have shown that As in one-, two-, or three-nucleotide bulges stack into the duplex [3], and generate a local bend [4], or a kink in the duplex DNA [20]. Purines, in general, prefer to stack in multi-nucleotide bulges [39,40] and in contrast to pyrimidines [2,37], prefer to stack within the duplex in one-base bulges [12,13].

The bulges were also studied by fluorescence spectroscopy, which is a useful technique to probe the solvent exposure of chromophores [14,41]. The fluoroside **Ç$^f$** (Fig. 1) has already shown its usefulness in studying nucleotide conformations in hairpin loops [29]. A potential pitfall is flanking-sequence dependence on the

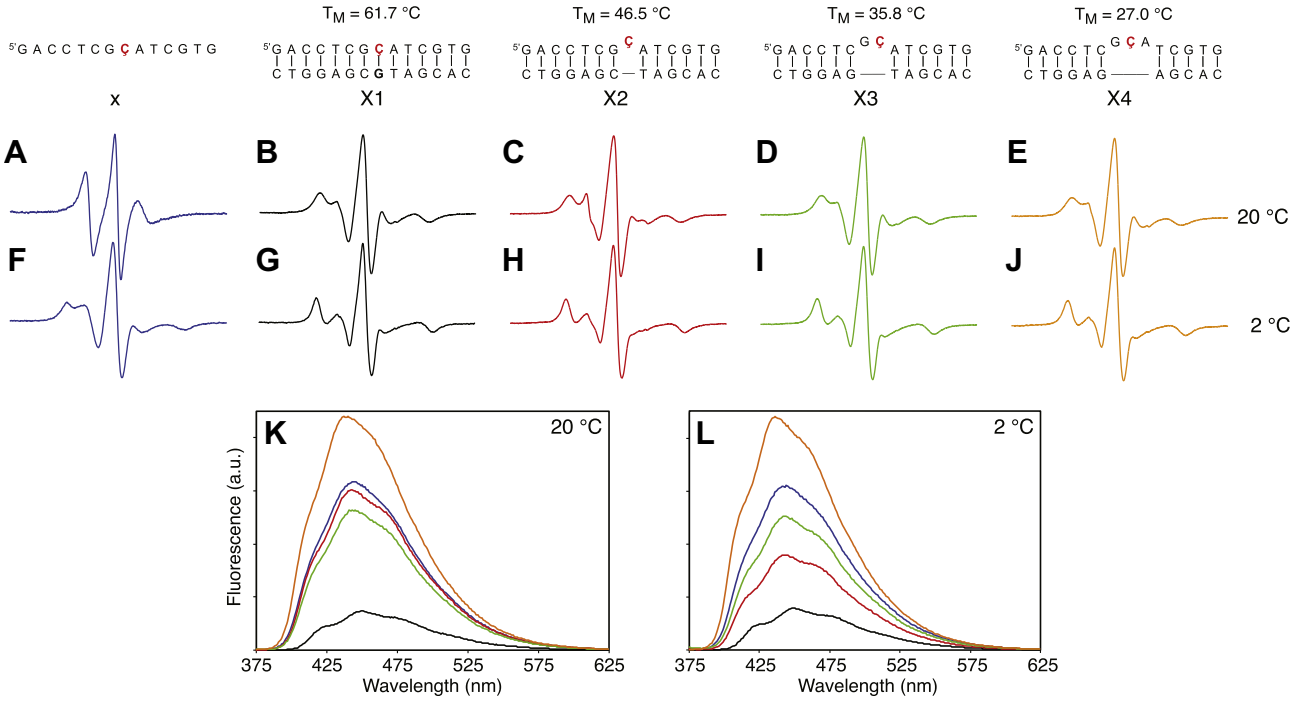


**Fig. 2.** EPR spectra of single-stranded oligomer **x** (A, F, blue), fully base-paired duplex **X1** (B, G, black), one-base bulge **X2** (C, H, red), two-base bulge **X3** (D, I, green) and three-base bulge **X4** (E, J, orange) were collected at 20 and 2 °C. Fluorescence spectra (K, L) of **x** and **X1**–**X4**, which are shown using the same color-code as for EPR spectra, were collected at the same temperatures. $T_M$ data of duplexes **X1**–**X4** are shown above the sequences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fluorescence signal, which has been observed with **Ç**$^{\mathbf{f}}$ [42]. However, the flanking sequence is the same in all the bulges and the fluoroside should, therefore, report on the solvent exposure of **Ç**$^{\mathbf{f}}$.

As expected, the fluorescence intensity of all three bulges at 2 and 20 °C was considerably higher than of the fully base-paired duplex, because a nucleotide in a DNA bulge is more exposed to the solvent than one that is placed in a duplex region (Fig. 2). Fluorescence of the bulges not only increases with size, but the fluorescence of the three-base bulge is also much higher than of the single strand. This indicates that the kink in the duplex makes **Ç**$^{\mathbf{f}}$ more solvent-exposed than when placed in the single strand, where it may be partially stacked. This is corroborated by NMR studies of the bulge sequences AAA [3], ATA [4], AAAAA [39] and AATAA [40], all of which have the nucleotides of the bulges localized at intrahelical positions within the double helices. This induces a local bend in the bulged DNA and exposes the bulged nucleotides to the solvent.

When lowering the temperature to 2 °C, the fluorescence of the one-base bulge dropped substantially, while the fluorescence of the other sequences remained unchanged. Thus, both the fluorescence and EPR data showed that the equilibrium between a stacked and a looped-out nucleotide is temperature-dependent and that stacking of **Ç**/**Ç**$^{\mathbf{f}}$ is favored at low temperature. In contrast, a cytidine one-base bulge, flanked by two Gs has shown the inverse trend and was predominantly looped-out at 0 °C [43]. NMR studies have also shown that flanking sequences have an effect on temperature-dependent equilibria of some one-base bulges [1]. Not all one-base bulges have temperature-dependent transitions, for example C was found to be looped-out at different temperatures when flanked by two As, presumably because of the propensity of the As to stack together [2].

### 3.2. Symmetrical internal loops

Having established that **Ç** and **Ç**$^{\mathbf{f}}$ were useful probes to investigate conformation and dynamics of nucleotides in bulges, we extended this study to the more flexible internal loops. Although the thermodynamic stability of small internal loops has been studied [44], little is known about their conformational dynamics. To systematically study internal loops, a family of duplexes (**Y1**–**Y9**) was prepared (Fig. 3). All the duplexes contained the central sequence 5′-d(CT**Ç**TC); the loop structure was changed by incorporating Gs (Fig. 3, bold) into the pyrimidine-rich opposing strand. As expected, thermodynamic stability of the duplexes increases as the size of the loops decreases (Fig. 3). Although internal loops are considerably less stable than duplex regions (for example, five-base loop **Y2** is ca. 28 °C less stable than duplex **Y1**) they are significantly more stable than asymmetric internal loops of the same length [44].

The EPR spectra of duplexes **Y1**–**Y9** and single-stranded DNA (**y**) were collected at 20 and 37 °C. Of all the EPR spectra collected at 20 °C, only the EPR spectrum of the five-base bulge **Y2** is slightly different, showing a more pronounced fast-motion component than the other spin-labeled oligomers (Fig. 3). However, the EPR spectrum of **Y2** is more similar to the spectrum of the fully base-paired duplex **Y1** than to the single strand (**y**) or even the one-base bulge **X2** (Fig. 2C). Thus, the mobility of **Ç** is severely restricted in the bulge, presumably due to stacking of the probe within the loop. The EPR spectra of all the other bulges (**Y3–Y9**) are identical to those of the fully base-paired duplex (**Y1**), showing that these loops are not very flexible at 20 °C and indicative of stacking within the internal loop.

In contrast to the EPR data at 20 °C, the EPR spectra of the duplexes at 37 °C reflect a greater variation in spin-label mobility (Fig. 3 and Fig. 4A). As a more quantitative measure of the mobility of **Ç** in different loops (**Y2**–**Y9**), we plotted the ratio of the height of the center peak of EPR spectrum ($h_c$) and the low-field peak ($h_l$) for all the spectra, except for the single strand (**y**) (Fig. 4A) [31]. Because $h_l$ corresponds to the fast-motion component, a high $h_l/h_c$ ratio reflects high spin-label mobility. This analysis revealed that oligomers **Y1–Y9** fall into three mobility categories. Duplex **Y2**, which contains the largest internal loop shows the most mobility

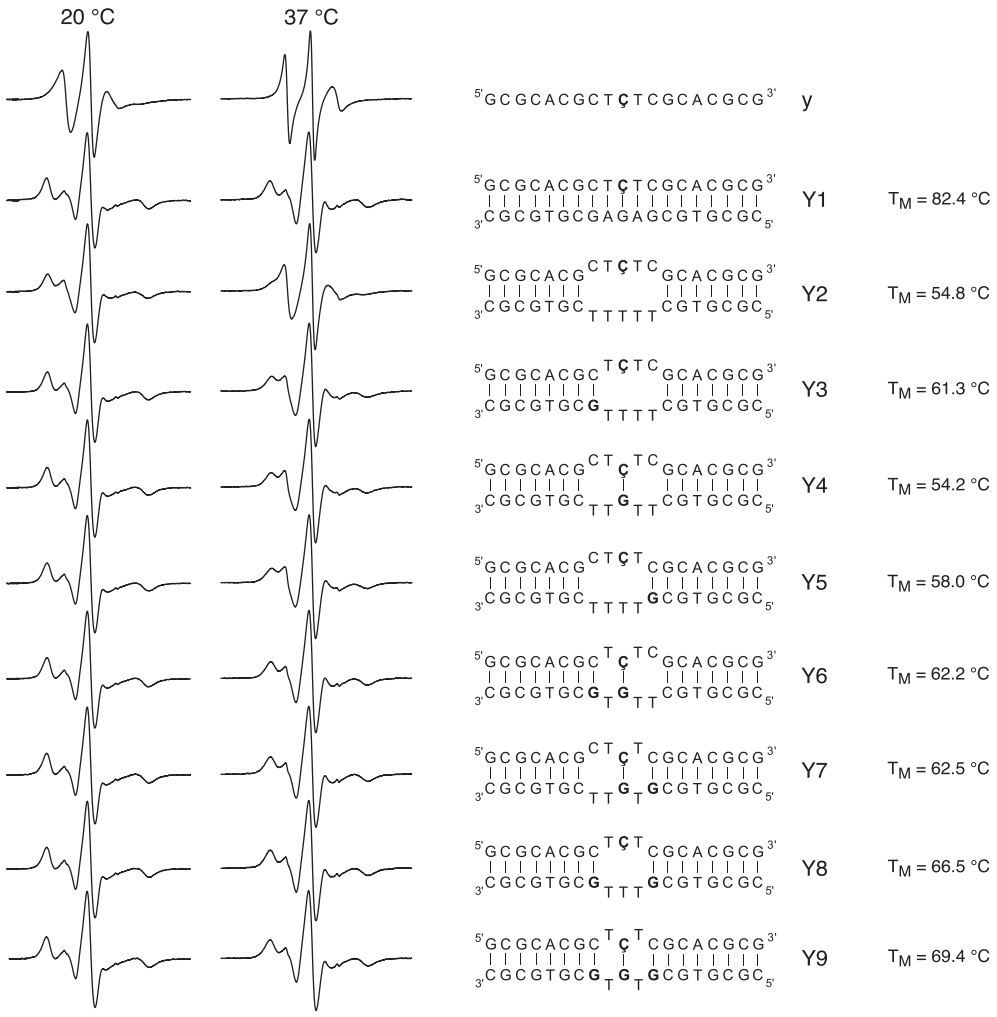


**Fig. 3.** EPR (left) and $T_M$ data (right) of internal loops **Y2**–**Y9**. EPR spectra of single strand **x**, fully base-paired duplex **Y1** and internal loops **Y2**–**Y9** in aqueous solution at 20 and 37 °C. The size of the loop was systematically varied by introducing Gs (shown bold) into the loop.

and is in a category by itself, displays spin-label mobility similar to the single strand (**y**). The second category contains duplexes **Y3**–**Y5**, all of which contain four mismatched pairs in the loop. The position of the additional base-pair, compared to **Y2**, does not appreciably affect the spin-label mobility. Duplexes **Y6**–**Y9** belongs to the third category, containing three or fewer bases in the loop. This category of loops, with the lowest $h_l/h_c$ ratio, shows the most extensive immobilization of **Ç** and has mobility similar to that of the duplex (**Y1**). Thus, EPR data of duplexes **Y1**–**Y9** at 37 °C shows a clear correlation between the $h_l/h_c$ ratio and a number of bases that are unpaired in the internal loop; the fewer unpaired bases in the loop, the lower the mobility of the probe.

The fluorescence quantum yields were also determined for duplexes **Y1**–**Y9** after reduction of **Ç** to **Ç$^f$**. The results for each construct at 2 and 37 °C are shown in Fig. 4B. For 37 °C, there is no obvious trend visible, in contrast to the EPR data. However, the fluorescence of the fully base-paired duplex **Y1** is considerably lower than for all other constructs, showing that fluorescence increases dramatically when **Ç$^f$** is placed in the loops. It is also noteworthy that loop **Y2**, which has the highest mobility among the internal loops as judged by EPR (Fig. 4A), has a substantially lower fluorescence at 2 °C than at 37 °C, indicating that base-stacking is favored in large internal loops at lower temperatures.

A clear trend was observed for the internal loops in duplexes **Y2**–**Y9** at 2 °C, where the fluorescence increased as the size of the loop decreased. Since the EPR data showed that the label was fully stacked within the duplex at 20 and 0 °C (data not shown), the fluorescence most likely reflects changes in the structure/dynamics of the loop. However, it is surprising that the fluorescence increased with decreased size of the loop. One might have expected the fluorescence to decrease with decreasing loop size, since the fluorescence of the fully base-paired duplex (**Y1**) is by far the lowest.

It is particularly striking that **Y8** and **Y9** show higher fluorescence than the single strand (**y**), which was also observed for the three-base bulge (**X4**). This data indicates a considerable local structural deformation of these loops from regular B-DNA that increases the exposure of the label to the solvent. NMR studies of single-base mismatches have shown that a single T·T mismatch is present as a wobble pair in duplex DNA; two hydrogen bonds are formed between carbonyls and imino protons, which causes only a minor distortion of the duplex structure [45]. Thus, it is unlikely Ts are looping out, although it cannot be ruled out, since the NMR studies were performed using a high salt concentration to stabilize the duplex structure. If a T·T mismatch that is positioned between a duplex stem and **Ç$^f$** is a dynamic equilibrium between stacked and looped-out conformation, it could lead to conformations where it is exposed to the solvent and lead to increased fluorescence. Our data show that duplexes **Y6** and **Y7**, both of which can form one T·T mismatch that is positioned between a duplex

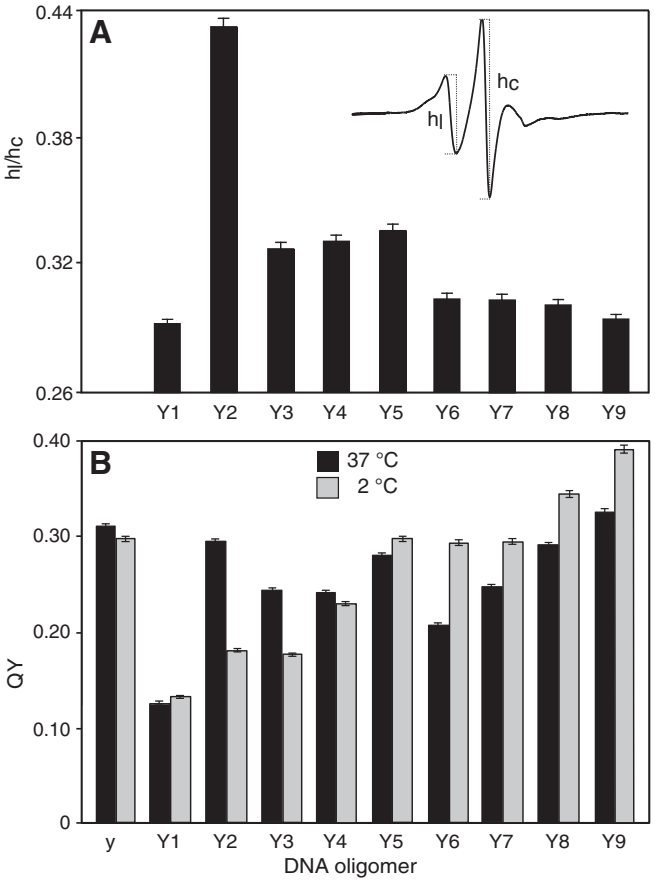


**Fig. 4.** (A) Mobility of **Ç** in **Y1**–**Y9** at 37 °C quantified as the $h_l/h_c$ ratio. Determination the $h_l/h_c$ ratio from the EPR spectrum; $h_l$ is the height of the low-field peak, $h_c$ is the height of the center peak. (B) Fluorescence quantum yield of single-stranded 19-mer **y**, fully base-paired duplex **Y1** and internal loops **Y2**–**Y9** at 37 °C (black) and 2 °C (gray).

stem and the label, have fluorescence similar to that of a single strand while **Y9** has two such T·T mismatches and has ca. 30% higher fluorescence than the single strand.

In conclusion, conformational dynamics of bulges and internal loops in duplex DNA were studied using the complementary techniques of EPR and fluorescence spectroscopy. The EPR and fluorescence data of the bulged DNAs show that stacking of the nucleotides is highly favored in all three bulges at both temperatures, except for the one-base bulge, which is present in temperature-dependent conformational equilibrium between looped-out and stacked states. The fluorescence of the larger bulges increased with increased size of the bulge, consistent with bending of the duplex and greater exposure of the fluorescent nucleoside to the solvent. EPR results of the symmetric internal loops did not show evidence of a looped-out conformation for the spin-labeled nucleotide at 20 °C, but the mobility of the nucleotides in the loop increased as the loop became larger at 37 °C. Fluorescence was unexpectedly highest for the smallest internal loops that had the lowest mobility by EPR, indicating local structural perturbation from B-form DNA. Thus, the complementary methods of EPR and fluorescence spectroscopies yield more information than either individual technique alone.

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