Chapter 4C

DNA-Binding Characteristics of Polyamide-Fluorophore Conjugates

Abstract

The successful staining of the nuclei of mammalian cells with polyamides labeled with fluorescein and other similar dyes permits many transcription inhibition experiments to be attempted with confidence that a negative result is not due to exclusion of active compound from genomic DNA. However, accompanying the need for good cellular uptake properties is the need for polyamides or polyamide conjugates to possess good DNA-binding properties. The effect of fluorophore conjugation on the affinity and specificity of polyamides for DNA is an important question pertaining to the use of these compounds as transcriptional regulators.

Several polyamide-fluorophore conjugates have been synthesized and their DNA-binding properties tested by DNase I footprinting titration experiments. In general, fluorescein conjugation is most tolerated at the *C*-terminal tail, resulting in a 5- to 25-fold decrease in affinity while retaining specificity. The effects of attachment off of the chiral turn residue and the N-terminal imidazole 4-amino group were also probed. Fluorescein conjugation to either of these positions resulted in a ~100-fold decrease in binding affinity with reference to the tail conjugate. Surprisingly, attachment of a Bodipy fluorophore from the turn residue results in a somewhat lesser 10-fold decrease in affinity, as compared to the tail-linked fluorescein conjugate.

Results

Compounds **1-6** were synthesized, linking the fluorescein moiety through the polyamide *C*-terminal tail (Figure 4.23). These polyamides target several different DNA binding sites, and were all shown to stain the nuclei of several different cell lines. Polyamides **1-3** were footprinted on a plasmid derived from the γ-globin promoter (Figure 4.24).

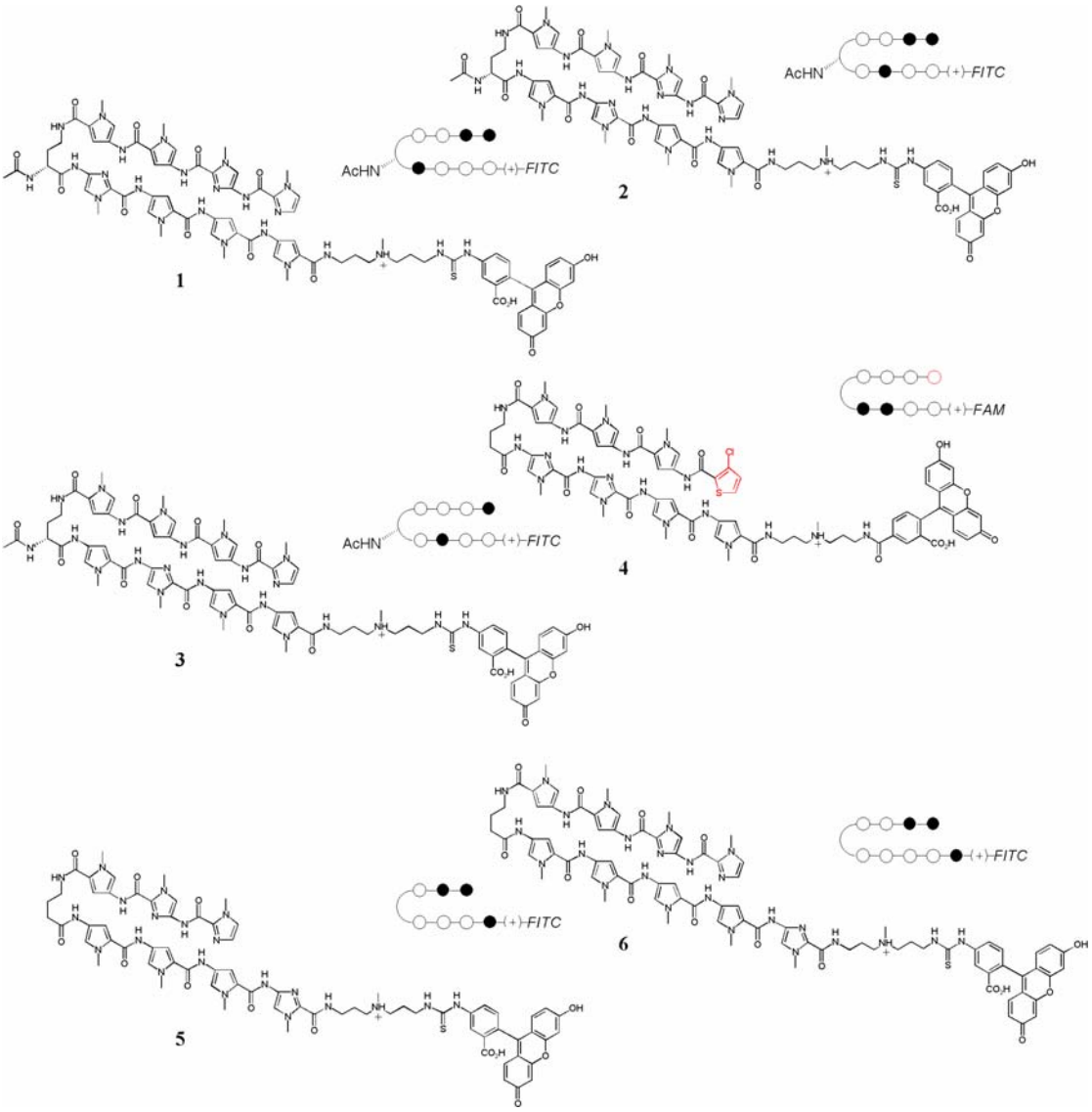

Figure 4.23 Chemical structures and ball-and-stick models of tail-linked polyamide-FITC conjugates **1-6**.

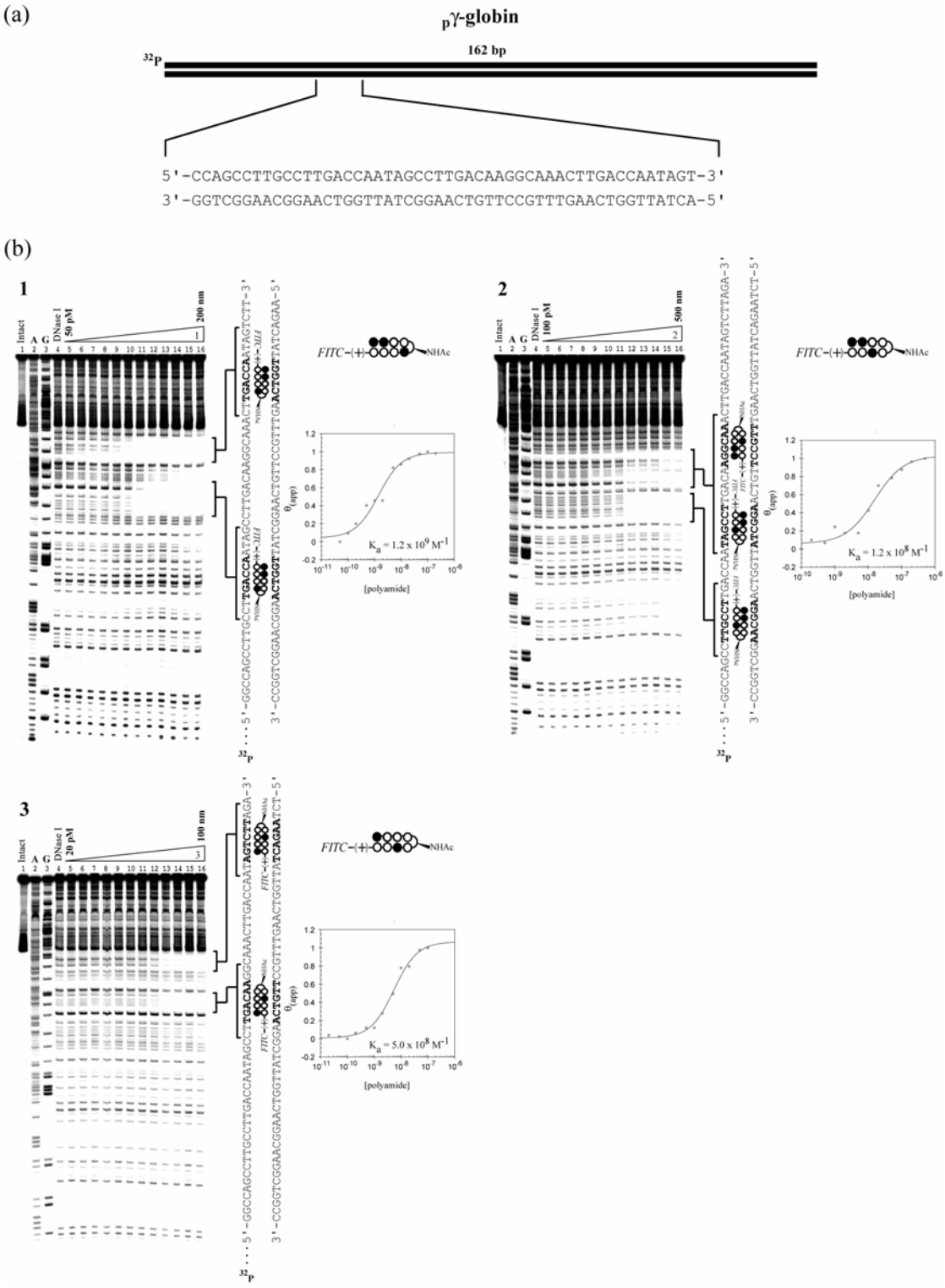

Figure 4.24 Footprinting of **1-3**. (a) γ-globin promoter PCR fragment used for footprinting reactions. (b) Footprinting gels and isotherms for compounds **1-3**.

The affinities of compounds **1**-**3** are reduced with reference to polyamides lacking the fluorescein moiety. The precise degree of this reduction is complicated by two factors. First, it is unclear which are the best choices of parent polyamides against which to compare binding characteristics. Second, for truly accurate comparisons to be made, both the polyamide and its conjugate must be footprinted in exactly the same sequence context, preferably on the same plasmid. Though imperfect, it is possible to make useful comparisons between the footprinting data reported here and past data.

Table 4.3 shows comparative data between compounds **1**-**3** and related polyamides **7**-**9**. There is a 4- to 25-fold decrease in binding affinity upon addition of the fluorescein moiety. The large differences in affinity decrease between compounds is most likely due to the imperfect comparisons that may be drawn between **1**-**3** and **7**-**9**, as well as the differing DNA contexts. However, the general effect of FITC addition seems to be an approximately 10-fold reduction in affinity, with good specificity.

Table 4.3 Comparison between the DNA-binding affinities of polyamides and polyamide-FITC conjugates.

	polyamide-FITC conjugate	K_a (M^{-1})		*polyamide*	K_a (M^{-1})
1	5'-at**TGGTCA**ag-3'  5'-ta**ACCAGT**tc-3'	1.2×10^9	**7**	5'-ta**TGGTCA**tg-3'  5'-at**ACCAGT**ac-3'	4.8×10^9
2	5'-ca**AGGCAA**gg-3'  5'-gt**TCCGTT**cc-3'	1.2×10^8	**8**	5'-gc**AGGCAA**cc-3'  5'-cg**TCCGTT**gg-3'	3.0×10^9
3	5'-ct**TGTCAA**gg-3'  5'-ga**ACAGTT**cc-3'	5.0×10^8	**9**	5'-gg**AGTCTA**ta-3'  5'-cc**TCAGAT**at-3'	2.0×10^9

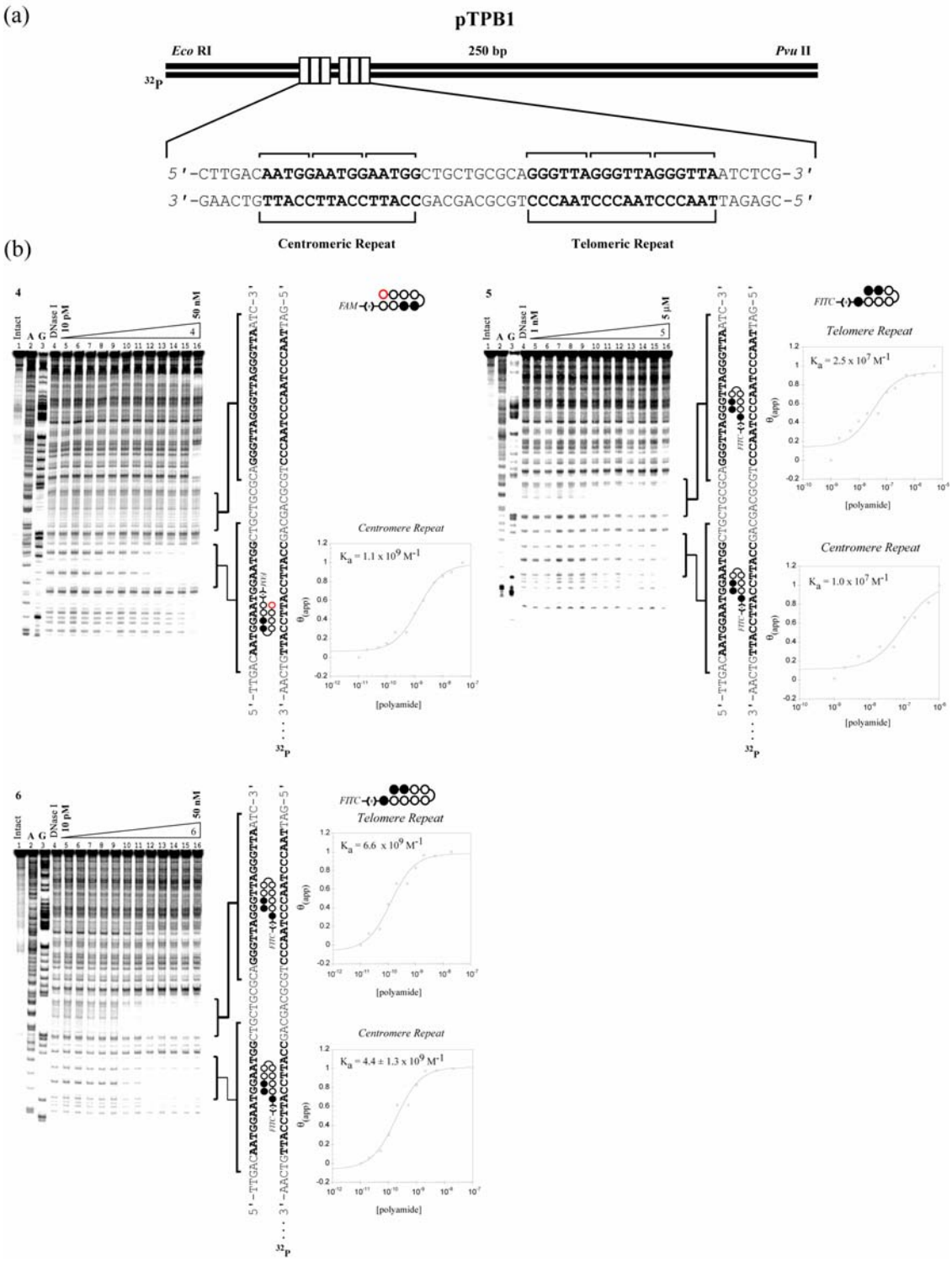

Figure 4.25 Footprinting of **4-6**. (a) pTPB1 restriction fragment used for footprinting reactions. (b) Footprinting gels and isotherms for compounds **4-6**.

The affinities of compounds **4** and **6** are similar in magnitude to those of compounds **1**-**3**, while the affinity of 7-ring compound **5** is substantially lower, probably reflecting a parent polyamide with poor affinity (Figure 4.25). Though their affinities are almost certainly reduced with reference to polyamides lacking the fluorescein moiety, there is no footprinting data available for likely parent polyamides. As for compounds **10**-**13**, they all are related to compound **7**, to varying degrees, and even more closely to compound **1**, differing mainly in the placement of the fluorophore moiety (Figure 4.26). Only compound **10**, which is structurally very similar to **1**, possesses a low nanomolar binding affinity. None of the other compounds have affinities as high as **1**, though surprisingly the Bodipy compound **13** has a 10-fold higher affinity than either **11** or **12**, which are both 100-fold lower than **1** (Figure 4.27). It should be noted that while compounds **10** and **13** were footprinted on the plasmid pDEH9, **11** and **12** were footprinted on the γ-globin plasmid (Figure 4.24a).

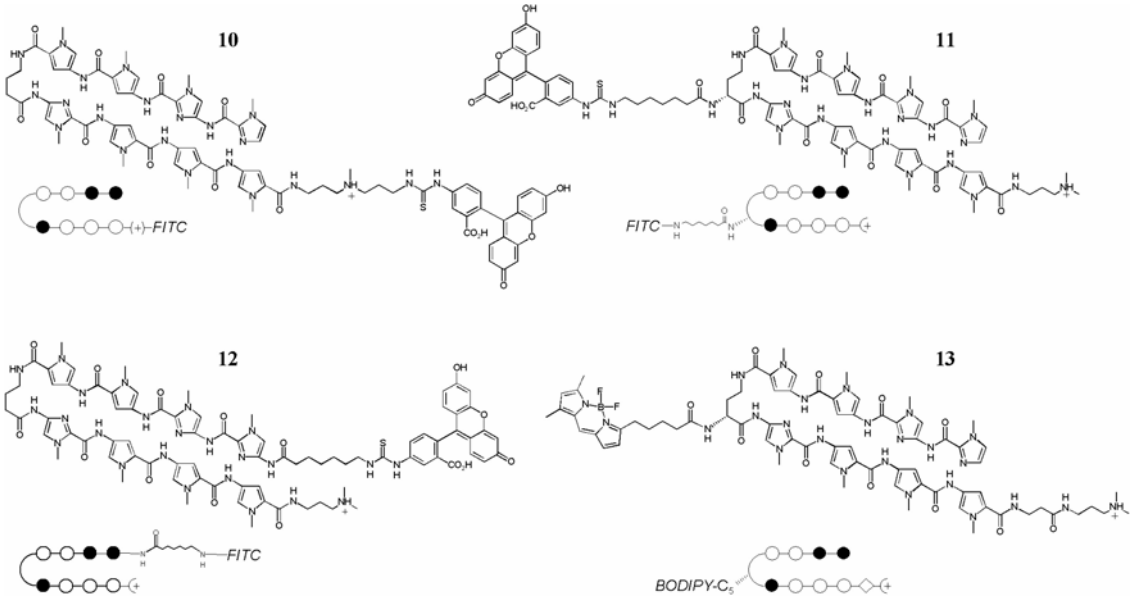

Figure 4.26 Chemical structures and ball-and-stick models of compounds **10**-**13**.

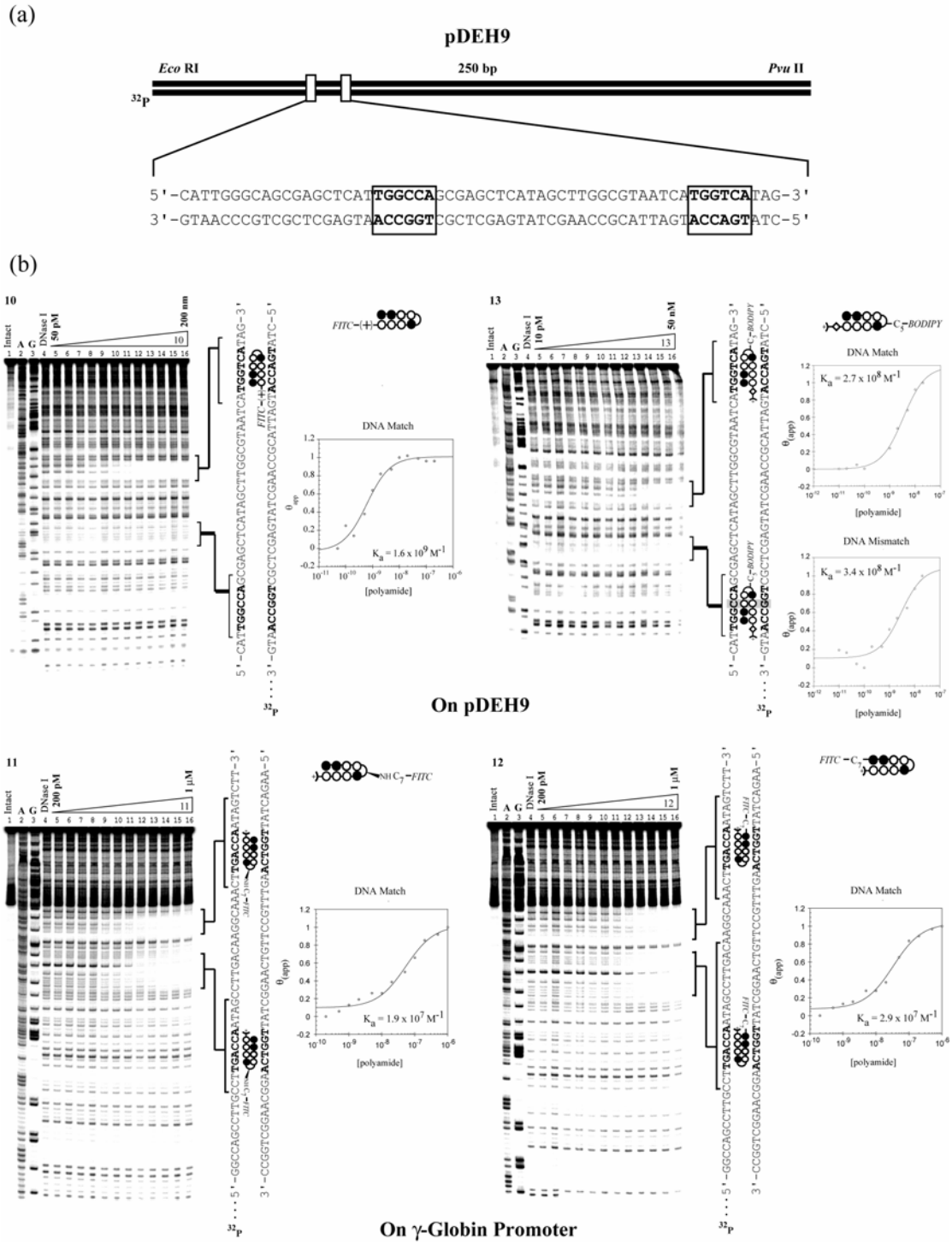

Figure 4.27 Footprinting of **10**-**13**. (a) pDEH9 restriction fragment used for footprinting reactions. (b) Footprinting gels and isotherms for compounds **10**-**13**.

Table 4.4 Comparison of alternate motifs for polyamide-FITC conjugates.

	compound	K_a (M^{-1})
7	5'-ta**TGGTCA**tg-3' 5'-at**ACCAGT**ac-3'	4.8×10^9
1	5'-at**TGGTCA**ag-3' FITC, NHAc 5'-ta**ACCAGT**tc-3'	1.2×10^9
10	5'-ca**TGGTCA**ta-3' FITC 5'-gt**ACCAGT**at-3'	1.6×10^9
11	5'-at**TGGTCA**ag-3' FITC 5'-ta**ACCAGT**tc-3'	1.9×10^7
12	5'-at**TGGTCA**ag-3' FITC 5'-ta**ACCAGT**tc-3'	2.9×10^7
13	5'-ca**TGGTCA**ta-3' BODIPY 5'-gt**ACCAGT**at-3'	2.7×10^8

An examination of the binding affinities of compounds **10**-**13** in comparison with those of **1** and **7** show that attachment of the fluorophore moiety from either the turn or the N-terminal imidazole 4-amino- group causes a large reduction in binding affinity. Attachment of FITC from the N-terminus results in 100- to 1000-fold reduction of affinity with reference to a parent polyamide. A comparable affinity reduction is seen when FITC is attached from the chiral turn via a C_7 aliphatic linker. It turns out that this affinity can be improved by attaching Bodipy in this position, via a C_5 aliphatic linker, for an overall 10- to 100-fold reduction in affinity with reference to polyamide. Attachment of these fluorophores from alternate positions seems to lead to a loss of sequence specificity in addition to the affinity drop. In general, this bodes poorly for using polyamide-fluorophore conjugates connected through alternate strategies as reagents to regulate gene transcription in living systems, despite the ready nuclear localization of compounds **11**-**13**.

Experimental

Chemicals

All polyamide precursors to **1**-**6** and **10**-**13** were synthesized on either Boc-β-Ala-PAM resin or Kaiser oxime resin, as previously described.[1,2] Polyamides were labeled with fluorophores by treatment with fluorescein isothiocyanate (1.5 eq) and DIEA (>25 eq) in DMF (100-200 μL).

DNase I Footprinting Titrations

5' or 3' ^{32}P-labeled PCR fragments were generated from template plasmid p γ-globin, pDEH9, or pTPB1 in accordance with standard protocols and isolated by nondenaturing gel electrophoresis.[3] All DNase I footprinting reactions were carried out in a volume of 400 μL. A polyamide stock solution or water (for reference lanes) was added to TKMC buffer, with final concentrations of 50 mM Tris-HCl, 50 mM KCl, 50 mM $MgCl_2$, and 25 mM $CaCl_2$, pH 7.0, and 15 kcpm 5'-radiolabeled DNA. The solutions were equilibrated for 12-18 h at 22°C. Cleavage was initiated by the addition of 10 μL of a DNase I stock solution and was allowed to proceed for 7 min at 22°C. The reactions were stopped by adding 50 μL of a solution containing 2.25 M NaCl, 150 mM EDTA, 0.6 mg/mL glycogen, and 30 μM base pair calf thymus DNA and then ethanol precipitated. The cleavage products were resuspended in 100 mM Trisborate-EDTA/80% formamide loading buffer, denatured at 85°C for 10 min, and immediately loaded onto an 8% denaturing polyacrylamide gel (5% cross-link, 7 M urea) at 2000 V for 2 h and 15 min. The gels were dried under vacuum at 80 °C and quantitated using storage phosphor technology.

References

1) Baird, E.E.; Dervan, P.B. *J. Am. Chem. Soc*. **1996**, *118*, 6141.

2) Belitsky, J.M.; Nguyen, D.H.; Wurtz, N.R.; Dervan, P.B. *Bioorg. Med. Chem.* **2002**, *10*, 2767.

3) Trauger, J.W.; Dervan, P.B. *Methods Enzymol*. **2001**, *340*, 450.