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A Binary Deoxyribozyme for Nucleic Acid Analysis

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Simple, sequence-specific, and sensitive methods for DNA/RNA analysis are required for the rapid diagnosis of infection and genetic diseases, genome study, mRNA monitoring in living cells as well as environmental and forensic applications. The aim of this work is to introduce a new approach for the design of a highly selective probe for nucleic-acid detection based on deoxyribozyme molecules. The formation of at least 15–20 nucleotide-long hybrids between probe and analyte is required to uniquely define a specific fragment in a nucleic acid mixture of the genome size. Hybrids of such length are too stable to be sensitive to base mispairing since a single mismatch unit results in a relatively small energetic penalty.[1] Conventional techniques that use buffers with low ionic strength, denaturing agents, or elevated temperatures do not always lead to the desirable selectivity.[1,2] One approach to improve selectivity of nucleicacid hybridization was realized with conformationally constrained probes, such as molecular beacons (MBs).[3,4] MBs are oligo-eoxyribonucleotide hairpins with a fluorophore and quencher conjugated to the opposite ends of the oligomer. Binding to complementary nucleic acids switches MBs to the elongated conformation and increases their fluorescence. MBs distinguish mismatches over a wider temperature range than unconstrained probes do, because the stem–loop structure stabilizes the probe–analyte dissociated state.[4] Alternatively, the specificity of nucleic-acid recognition can be increased by splitting the probe into two halves.[5] Binary probes are more selective than conventional probes because each relatively short hybrid (7–10 nucleotides) is extremely sensitive to single-base mispairings. Here, a binary probe based on a deoxyribozyme that contains structural constrains is designed for the recognition of single-base substitutions in 20-mer DNA analytes, at room temperature.

Deoxyribozymes, or DNA enzymes, are catalytic oligodeoxyribonucleotides derived by in vitro selection.[6] The advantages offered by catalytic DNAs include high chemical stability, low cost for synthesis, biocompatibility, and ease of structural prediction and modification. This relatively new class of catalytic molecules has been considered as a promising biochemical tool for nucleic-acid detection.[7] One particular attraction of DNA enzyme-based probes is their potentially improved sensitivity due to the catalytic amplification of the positive signal. Therefore, binary probes based on deoxyribozymes promise to be both highly selective and sensitive. Deoxyribozyme E6, which was selected earlier by Breaker and Joyce,[8] was chosen in this work as a model for proof-of-concept experiments.

Deoxyribozyme E6 (Figure 1A) is a Mg^{2+}-dependent DNA enzyme that recognizes DNA substrate with a single embedded ribonucleotide, and hydrolyzes the RNA phosphodiester bond

with a catalytic rate of ~0.01 min^{-1}.[8] It has been shown that E6 is able to cleave a fluorophore- and quencher-labeled substrate with approximately the same rate.[7b] This fluorescence-based approach has been used as the most convenient method for monitoring E6 catalytic activity. E6 contains a variable stem–loop, which serves only a structural function and is not directly involved in catalysis; this allowed the design of the binary probe.

Deoxyribozyme E6 was divided into two fragments (biE6a and biE6b), the inessential AAG loop was removed, stem 1 was elongated to six nucleotides, and the analyte-binding arms were added to each half with dithymidine linkers (Figure 1 B). Structural constraints in the form of two pentanucleotide stems (stem 2 and 3) were introduced in the analyte-binding arms to further increase the selectivity of the binary probe.[5e] The reporter substrate (F substrate; Figure 1C) used in the study was complementary to the substrate-binding arms of the deoxyribozyme and contained a fluorophore and quencher at its 5'- and 3'-ends, respectively. When the nucleic-acid analyte was added, the two subunits of the enzyme cooperatively hybridized to the complementary region of the analyte and re-formed the deoxyribozyme catalytic core (Figure 1C). The active enzyme cleaved the reporter substrate; this led to higher fluorescence (Figure 1 C).

It was found that addition of 80 n$_M$ A20 DNA analyte to a solution of biE6 and F substrate triggered an approximately four-times increase in fluorescence after 1 h incubation (Figure 2 A, graph 2). The rate of fluorescence increase was about the same when 80 n$_M$ E6 was incubated with F substrate (graph 4). Therefore, 80 n$_M$ A20 generated about 80 n$_M$ active biE6 in solution according to the suggested scheme (Figure 1 C). At the same time, biE6 activity was not observed in the absence of A20 analyte (graph 1). Polyacrylamide gel electrophoresis (PAGE) of the reaction mixtures revealed cleavage of F substrate in reaction mixtures 2 and 4 (Figure 2B, lanes 2 and 4), but not in control samples 1 and 3 (lanes 1 and 3). The observed intensities of the cleavage product in samples 2 and 4 were about the same; this was in good agreement with the fluorescent data (Figure 2A). These results prove the suggested model for analyte-dependent binary deoxyribozyme activation (Figure 1 C), and indicate that hybridization of the analyte-binding arms to A20 does not significantly reduce the catalytic activity of the core.

To investigate the selectivity of biE6 the probe was incubated with single-base substituted analogues of A20 analyte (Table 1). It was found that the binary deoxyribozyme distinguished nineteen mutants out of the twenty tested (column 3). The discrimination factors (DFs) for eleven of the oligodeoxyribonucleotides were significantly higher than 3 (marked bold in Table 1). Taking into account that a signal:-background ratio (S/B) >3–4 is considered acceptable for fluorescence-based assays,[9] I conclude that biE6 might be practically useful for the detection of at least some of these eleven mutations. In comparison, the conventional molecular-beacon approach did not provide such high selectivity: MB-A20 (FAM–5'-CTCGCACCC ACTCTCTCCATGCGAG–dabcyl), an anti-A20 molecular beacon, distinguished true target from only 14 single-base substituted oligonucleotides with low DFs (Table 1, last column). These results indicate that biE6 might be an efficient single nucleotide polymorphism typing tool even at room temperature.

In order to determine the sensitivity limit of the probe, biE6 was incubated with various concentrations of A20 analyte in the presence of F substrate. The ratios of the fluorescence intensities and background fluorescence as a function of the logarithm of analyte concentration are shown in Figure 3. In the presence of 1 n$_M$ analyte, the probe-generated fluorescence was more than three-times greater than the background (S/B 3.9). At the same time, the probe demonstrated high selectivity: 100 nm A20-4 gave a S/B ratio (1.9) that did not exceed the threshold of 3 (graph 2). In comparison, MB-A20 detected A20 only at a concentration of 20 n$_M$ (graph 3), while 100 n$_M$ A20-4 generated a detectable signal (S/B ~3; Figure 3, graph 4).

Thus, in the experiments with A20 and A20-4, biE6 was at least 20-times more sensitive and more selective than MB-A20.

Although binary hammerhead ribozymes[10] and binary deoxyribozyme ligases[11] have been reported, to the best of my knowledge, biE6 is the first split DNAzyme designed for fluorescent detection of specific nucleic acids. Excellent selectivity at room temperature is the main advantage of this probe. The binary construct based on E6 deoxyribozyme can detect 1 n$_M$ analyte. At the same time, split probes designed on the platforms of more efficient enzymes, such as DNAzymes recently obtained by Li and colleagues,[12] can potentially improve the sensitivity of this approach. The probe promises to be relatively inexpensive for multiplex analysis, since it requires synthesis of only two short unmodified DNA strands for each new analyte sequence while the double labeled reporter substrate is universal and can be utilized efficiently in bulk amounts. All these advantages raise the hope that further development of the technique will deliver a highly selective, sensitive, and inexpensive PCR-free method for nucleic-acid analysis.

Experimental Section

Materials

DNAse/RNAse free water was purchased from Fisher Scientific, Inc. (Pittsburgh, PA, USA) and used for all buffers and for the stock solutions of oligonucleotides. Oligonucleotides were custom made by Integrated DNA Technologies, Inc. (Coralville, IA, USA) and by TriLink BioTechnologies, Inc. (San Diego, CA, USA). Fluorescent spectra were measured by using a Perkin–Elmer (San Jose, CA, USA) LS–55 luminescence spectrometer with a Hamamatsu xenon lamp. Experiments were performed at an excitation wavelength of 485 nm and emission was monitored from 500 to 550 nm; data were processed by using Microsoft Excel.

Polyacrylamide gel electrophoresis (PAGE)

F substrate (200 n$_M$) and biE6 (100 n$_M$, each strand) were incubated in the absence or presence of A20 DNA analyte (80 n$_M$) in MgCl$_2$ (50 m$_M$), HEPES (50 m$_M$), pH 7.4 (samples 1 and 2). Control sample 3 contained F substrate (200 n$_M$) only; control sample 4 contained F substrate (200 n$_M$) and deoxyribozyme E6 (80 n$_M$). After 1 hr incubation at room temperature each sample (100 µL) was ethanol precipitated and centrifuged (10 min, 16100 RCF). The resulting pellets were dissolve in 90% formamide (5 µL) that contained EDTA(50 m$_M$), bromophenol blue (0.1 %), xylene cyanol (0.1 %), TBE buffer (1x), and analyzed by using a polyacrylamide gel (20%) containing urea (7$_M$). The gel was photographed, without staining, by using Alpha-imager 3400 (Alpha Innotech, San Leandro, CA, USA).

Discrimination factors

Solutions of F substrate (200 n$_M$) and biE6 (200 n$_M$ each strand) were incubated in the presence of A20 DNA analyte (80 n$_M$) or one of the single-base substituted oligodeoxyribonucleotides in MgCl$_2$ (50 m$_M$), HEPES (50 m$_M$), pH 7.4. Fluorescence-emission spectra were recorded after 2 h incubation at room temperature. Fluorescence intensities at 517 n$_M$ were used for the calculation of the discrimination factors.

Sensitivity experiments

F substrate (1000 n$_M$) and biE6 (100 n$_M$ each strand) were incubated with various concentrations of A20 or A20-4 in NaCl (1$_M$), HEPES (50 m$_M$), pH 7.4, ZnCl$_2$ (1 m$_M$), and split into six tubes (120 µL in each). Since biE6 showed ~ 15% higher efficiency in Zn^{2+}-containing buffer than in Mg^{2+}-containing buffer (data not shown) the former buffer was chosen for sensitivity experiments. Control samples used for measuring the background fluorescence did not contain the analyte or its analogues. Fluorescent-emission spectra were recorded after 30 h incubation

at room temperature. The ratio between the fluorescence intensities at 517 nm in the presence of A20 or A20-4 and the fluorescence intensity of the control sample were calculated. The average values of three independent experiments were plotted as a function of analyte concentration.

MB-A20 assay

Experiments with MB-A20 (FAM–5'-CTCGCACCCACTCTCTCCATGCGAG–dabcyl) were carried out in the buffer that was earlier optimized for the selective recognition of DNA analytes by a molecular beacon: KCl (100 m$_M$), MgCl$_2$ (1 m$_M$), Tris-HCl (10 m$_M$), pH 8.0.[4] For selectivity studies, a solution of MB-A20 (20 n$_M$) was incubated in the presence of either A20 (40 n$_M$) or one of the single-base substituted oligodeoxynucleotides. For sensitivity studies, a solution of MB-A20 (20 n$_M$) was incubated with various concentrations of either A20 or A20-4. The control sample did not contain A20 or A20-4. Fluorescent-emission spectra were measured after 15 min incubation at room temperature. The fluorescence intensities at 517 n$_M$ were measured for calculation of DFs and S/B ratios.

Acknowledgments

References

1. Demidov VV, Frank-Kamenetskii MD. Trends Biochem. Sci 2004;29:62–71. [PubMed: 15102432]

2. Urakawa H, El Fantroussi S, Smidt H, Smoot JC, Tribou EH, Kelly JJ, Noble PA, Stahl DA. Appl. Environ. Microbiol 2003;69:2848–2856. [PubMed: 12732557]

3. Tyagi S, Kramer FR. Nat. Biotechnol 1996;14:303–308. [PubMed: 9630890]

4. Bonnet G, Tyagi S, Libchaber A, Kramer FR. Proc. Natl. Acad. Sci. USA 1999;96:6171–6176. [PubMed: 10339560]

5. a) Xu Y, Karalkar NB, Kool ET. Nat. Biotechnol 2001;19:148–152. [PubMed: 11175729] b) Sando S, Abe H, Kool ET. J. Am. Chem. Soc 2004;126:1081–1087. [PubMed: 14746476] c) Bichenkova EV, Savage HE, Sardarian AR, Douglas KT. Biochem. Biophys. Res. Commun 2005;332:956–964. [PubMed: 15913549] d) Kolpashchikov DM. J. Am. Chem. Soc 2005;127:12442–12443. [PubMed: 16144363] e) Kolpashchikov DM. J. Am. Chem. Soc 2006;128:10625–10628. [PubMed: 16895431] f) Marti AA, Li X, Jockusch S, Li Z, Raveendra B, Kalachikov S, Russo JJ, Morozova I, Puthanveettil SV, Ju J, Turro NJ. Nucleic Acids Res 2006;34:3161–3168. [PubMed: 16769776]

6. a) Joyce GF. Annu. Rev. Biochem 2004;73:791–836. [PubMed: 15189159] b) Achenbach JC, Chiuman W, Cruz RP, Li Y. Curr. Pharm. Biotechnol 2004;5:321–336. [PubMed: 15320762]

7. a) Todd AV, Fuery CJ, Impey HL, Applegate TL, Haughton MA. Clin. Chem 2000;46:625–630. [PubMed: 10794743] b) Stojanovic MN, de Prada P, Landry DW. ChemBioChem 2001;2:411–415. [PubMed: 11828471] c) Sando S, Sasaki T, Kanatani K, Aoyama Y. J. Am. Chem. Soc 2003;125:15720–15720. [PubMed: 14677943] d) Tian Y, Mao C. Talanta 2005;67:532–537. [PubMed: 18970201] e) Sando S, Narita A, Sasaki T, Aoyama Y. Org. Biomol. Chem 2005;3:1002–1007. [PubMed: 15750642]

8. Breaker RR, Joyce GF. Chem. Biol 1995;2:655–660. [PubMed: 9383471]

9. Aslan K, Huang J, Wilson GM, Geddes CD. J. Am. Chem. Soc 2006;128:4206–4207. [PubMed: 16568977]

10. a) Iyo M, Kawasaki H, Taira K. Methods Mol. Biol 2004;252:257–265. [PubMed: 15017055] b) Vorobjeva M, Zenkova M, Venyaminova A, Vlassov V. Oligonucleotides 2006;16:239–252. [PubMed: 16978087]

11. Tabor JJ, Levy M, Ellington AD. Nucleic Acids Res 2006;34:2166–2172. [PubMed: 16648360]

12. a) Shen Y, Chiuman W, Brennan JD, Li Y. ChemBioChem 2006;7:1343–1348. [PubMed: 16888734]
b) Chiuman W, Li Y. Chem. Biol 2006;13:1061–1069. [PubMed: 17052610] c) Chiuman W, Li Y.
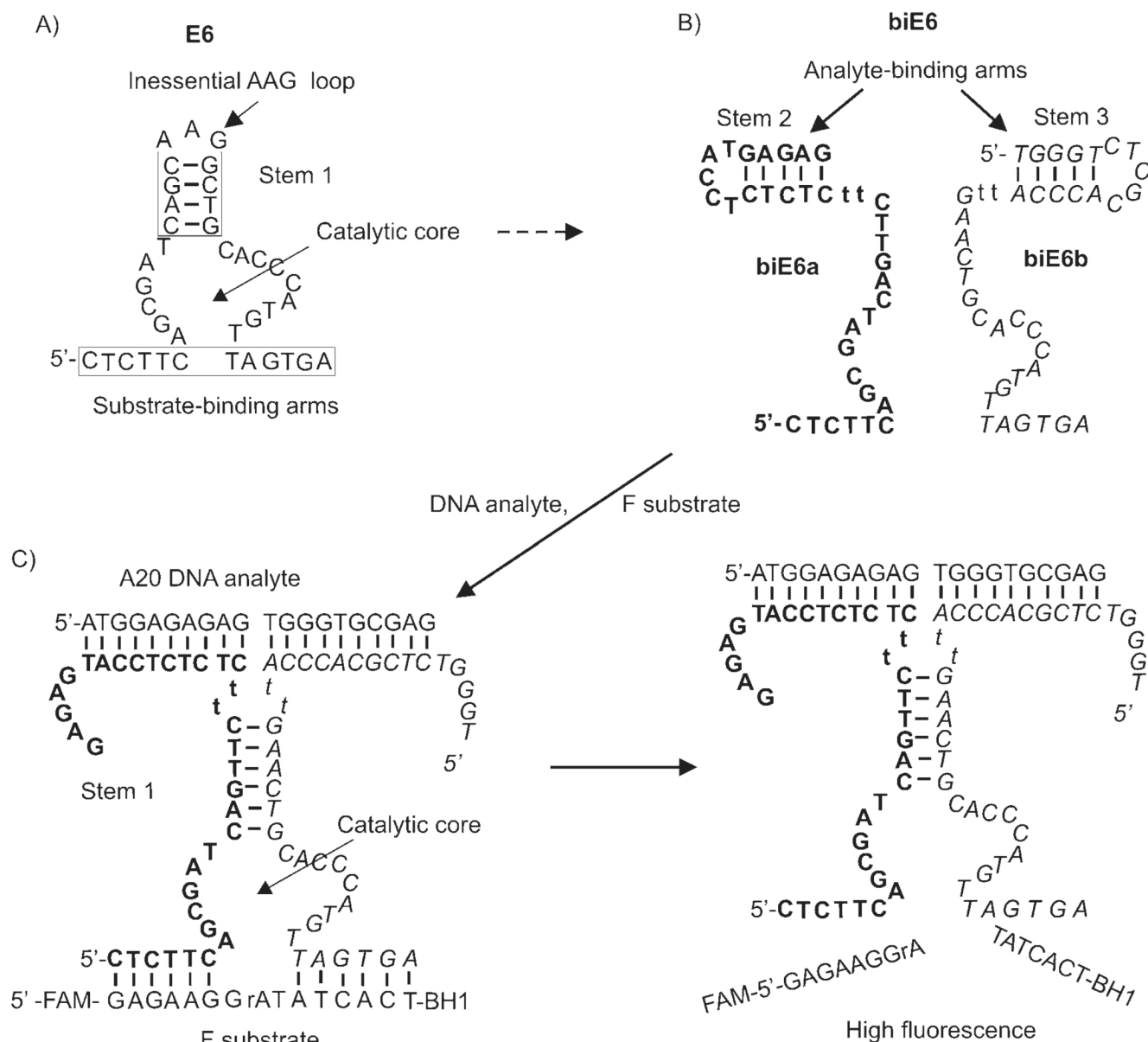J. Mol. Biol 2006;357:748–754. [PubMed: 16480741]

Figure 1.
Design of the binary deoxyribozyme probe. A) Structures of the parent deoxyribozyme E6.
[8] B) Binary deoxyribozyme biE6. C) Scheme for fluorescent detection of the analyte-
dependent catalytic activity of biE6. The dithymidine linkers are shown in lower case letters.
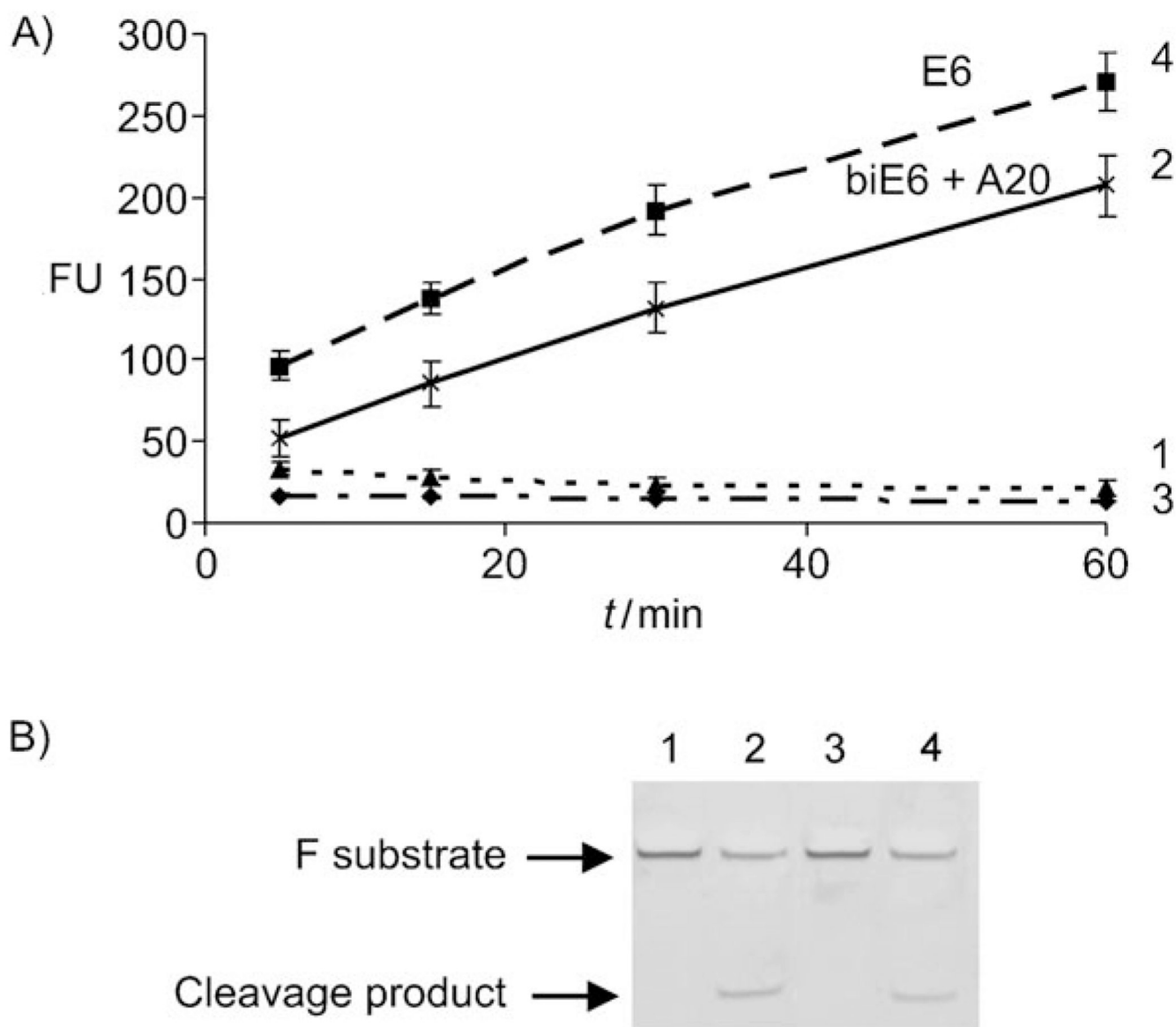FAM indicates fluorescein, BH1 is black hole quencher 1.

Figure 2.
Binary deoxyribozyme cleaves fluorogenic substrate only in the presence of A20 DNA analyte. F substrate (200 n_M) and biE6 (100 n_M, each strand) were incubated in the absence (sample 1) or presence (sample 2) of A20 DNA analyte (80 n_M) in $MgCl_2$ (50 m_M), HEPES (50 m_M), pH 7.4. Sample 3 contained F substrate only (control); sample 4 contained F substrate (200 n_M) and deoxyribozyme E6 (80 n_M). A) Fluorescent intensities (FU) at 517 nm are represented as a function of incubation time. The numbers next to the graphs correspond to the samples. Data are average values of three independent measurements. B) PAGE analysis of samples after 1 h incubation at room temperature. Lanes 1–4 correspond to samples 1–4, respectively. Only the 5'-end cleavage product, which contains the FAM group, was visible in the gel without staining.
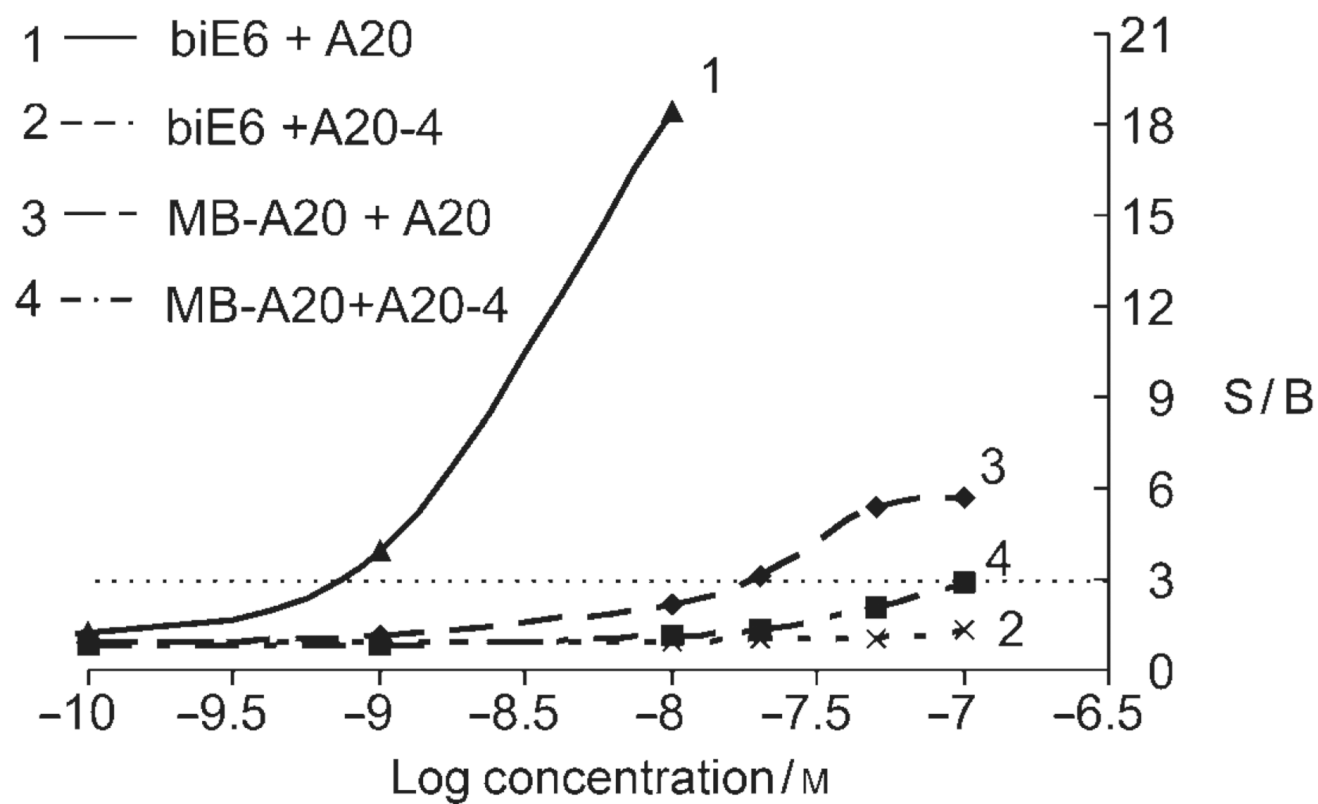
Figure 3.
Sensitivity of biE6 (graphs 1 and 2) in comparison to MB-A20 (graphs 3 and 4). Relative
fluorescence intensities at 517 nm are represented as functions of A20 (graphs 1 and 3) and
A20-4 (graphs 2 and 4) concentrations; S/B is signal:background ratio.

Table 1

Discrimination factors (DFs)[a] for oligodeoxyribonucleotides that differed from A20 by a single nucleotide.

Oligodeoxyribonucleotide analytes		biE6	MB-A20
name	sequence[b]		
A20	5'-ATGGAGAGAG TGGGTGCGAG	1	1
A20-1	5'-TTGGAGAGAG TGGGTGCGAG	**9.3±1.3**	2.1±0.6
A20-2	5'-AGGGAGAGAG TGGGTGCGAG	1.4±0.2	0.9±0.2
A20-3	5'-ATAGAGAGAG TGGGTGCGAG	**5.4±0.8**	1.1±0.1
A20-4	5'-ATGTAGAGAG TGGGTGCGAG	**43.7±3.2**	2.1±0.3
A20-5	5'-ATGGCGAGAG TGGGTGCGAG	**15.0±2.4**	2.3±0.6
A20-6	5'-ATGGATAGAG TGGGTGCGAG	**9.9±2.7**	1.2±0.1
A20-7	5'-ATGGAGGGAG TGGGTGCGAG	**3.6±0.5**	1.2±0.2
A20-8	5'-ATGGAGAAAG TGGGTGCGAG	**17.9±2.6**	1.6±0.1
A20-9	5'-ATGGAGAGCG TGGGTGCGAG	1.6±0.2	1.6±0.1
A20-10	5'-ATGGAGAGAT TGGGTGCGAG	3.0±0.4	1.1±0.1
A20-11	5'-ATGGAGAGAG GGGGTGCGAG	1.7±0.2	1.3±0.2
A20-12	5'-ATGGAGAGAG TAGGTGCGAG	2.7±0.6	1.1±0.1
A20-13	5'-ATGGAGAGAG TGTGTGCGAG	2.5±0.2	1.3±0.1
A20-14	5'-ATGGAGAGAG TGGTTGCGAG	**6.6±1.1**	1.2±0.1
A20-15	5'-ATGGAGAGAG TGGGAGCGAG	**13.0±1.9**	1.3±0.2
A20-16	5'-ATGGAGAGAG TGGGTTCGAG	3.0±0.2	1.2±0.1
A20-17	5'-ATGGAGAGAG TGGGTGTGAG	**9.3±1.3**	2.5±0.6
A20-18	5'-ATGGAGAGAG TGGGTGCCAG	**5.9±1.7**	1.3±0.2
A20-19	5'-ATGGAGAGAG TGGGTGCGGG	1.4±0.3	1.2±0.1
A20-20	5'-ATGGAGAGAG TGGGTGCGAA	0.9±0.1	1.1±0.1

[a]DFs were calculated as the ratios between probe fluorescence intensities at 517 nM in the presence of A20 (true target) and fluorescence intensities in the presence of each mismatched oligonucleotide, after subtraction of background fluorescence; mismatched positions are underlined. DFs that are greater than 3 are marked in bold; data are the average values of four independent measurements.

[b]The sequences of the 20 single-base substituted oligodeoxyribonucleotides were designed so as to introduce one substitution in every position; the type of each substitution was chosen randomly.