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# In Vitro Selection of Hematoporphyrin Binding DNA Aptamers

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**Abstract**—DNA aptamers that bind to hematoporphyrin IX (HPIX) were isolated using an in vitro selection technique. Most aptamers obtained after the 7th and 10th rounds contained guanine-rich sequences. Binding assay using fluorescence polarization technique and structural analysis by CD spectra revealed that the parallel guanine-quartet structure of the aptamer participates in the recognition of HPIX. © 2000 Elsevier Science Ltd. All rights reserved.

## Introduction

Porphyrins exist in various kinds of proteins, such as peroxidases, catalases, hemoglobins, photosynthetic proteins, cytochromes and so on. All of these proteins are crucial for life. In order to understand the reaction mechanism of the functional molecules, or to design new molecules that can perform the desired functions, many efforts have been made to mimic the porphyrin-proteins' functions using anti-porphyrin antibodies,[1] synthetic peptides[2] and organic compounds. The use of oligonucleotides for this purpose, however, has not been sufficiently studied.

The in vitro selection technique[3,4] has been used to isolate single-stranded nucleic acids (aptamers) that bind to a variety of targets, such as proteins and small molecules, with a high affinity.[5] Such aptamers have been shown to exhibit a variety of three-dimensional structures.[6] It has also been shown that some of the aptamers were able to catalyze chemical reactions.[7] Since aptamer selection takes place in vitro and does not require animals or cell lines, it is much faster, cheaper, and more versatile than antibody preparation. Additionally, oligonucleotides can be readily prepared and automatically reproduced by a DNA synthesizer much more quickly than can antibodies.

In the present study, we selected single-stranded DNAs (ssDNAs) which were capable of binding to hematoporphyrin IX (HPIX) with μM order of dissociation constants. Structural analysis of these aptamers indicated that guanine-rich (G-rich) sequences are necessary for recognition of HPIX. From the CD spectrum of the binding site of the aptamer, the structure of G-rich sequence is indicated to form the parallel guanine-quartet (G-quartet).

## In Vitro Selection of HPIX Aptamer

Immobilization of HPIX (Funakoshi Co.) on EAH Sepharose 4B (Amersham Pharmacia Biotech) was carried out according to the manufacturer's instruction. Synthetic DNA templates (103-mer) with 59 random nucleotides, 5′-TAGGGAATTCGTCGACGGATCC-N59-CTGCAGGTCGACGCATGCGCCG-3′, were amplified over 10 cycles of PCR (94 °C, 15 s; 55 °C; 15 s; 72 °C, 15 s) using the primers, 5′-TAATACGACTCAC-TATAGGGAATTCGTCGACGGAT-3′ (P1) and 5′-CGGCGCATGCGTCGACCTG-3′ (P2). ssDNAs were obtained from the double-stranded DNAs (dsDNAs) by additional 45 cycles of PCR using only P1 primer. The PCR products were purified by 8% polyacrylamide gel electrophoresis. The ssDNA pool in selection buffer (100 mM Tris–acetate pH 8.0, 200 mM sodium acetate, 50 mM potassium acetate, 25 mM magnesium acetate, 5% dioxane) was subjected to the HPIX immobilized gel (300 μL). Unbound ssDNAs were washed away by the selection buffer (5 mL). Bound ssDNAs were competitively eluted with HPIX solution (2 mL, 3 mM).

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Eluted ssDNAs were amplified with PCR and used as the input DNAs for the next selection. This process was defined as a 'round', and was repeated 10 times. In the first eight rounds, ssDNAs and immobilized HPIX were incubated for 30 min at room temperature before being washed, while in rounds 9 and 10 no incubation was carried out to obtain aptamers which have higher affinity to HPIX. The ability of the eluted DNAs was determined by comparing the amount of applied DNA and unbound DNA. The amount of DNA was monitored by UV absorption at 260 nm. The amount of bound DNA increased every round.

The PCR products obtained after the 7th and 10th rounds of selection were subcloned into pUC18, then introduced into *Escherichia coli* (DH5α). The plasmid DNA was isolated by alkaline lysis. Twenty four and 10 DNA sequences were randomly selected after 7th and 10th selection, respectively, and determined by a dye-terminator method using the ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems). All clones except one (clone **7**) after 7th round were found to contain guanine-rich (G-rich) sequences (Table 1). However, no characteristic consensus sequence was observed.

## Determination of Dissociation Constant

The binding affinity of arbitrarily selected 10 aptamers to HPIX was evaluated by a fluorescence polarization analysis.[8] The aptamers in the selection buffer (50 μM) were heated at 95 °C for 5 min and slowly cooled to room temperature. The aptamers were diluted sequentially from 50 μM to 0.1 nM, then were mixed with HPIX (1 μM) and incubated for 30 min at room temperature. Polarization was measured using the Beacon 2000 Fluorescence Polarization System (PanVera). The wavelength of excitation filter was 390 nm and that of emission filter was 620 nm. The apparent dissociation constant was determined from the dose–response curve as $K_d$ value by nonlinear regression analysis using Prism (GraphPad Software) (Fig. 1 and Table 1). Among the selected aptamers, the clone **26** (● in Fig. 1) had the lowest binding constant ($K_d = 1.3 \times 10^{-6}$ M). On the other hand, the clone **7** (◇ in Fig. 1), which contains a single guanine-repeated sequence, showed no appreciable increase in polarization (Fig. 1). An ssDNA (5′-ACCTGCGA-TAGGTAGTTTGTTTCTGTTGCCCTAGCGTCCG-TCGTAACAAGTCA-3′, □ in Fig. 1) which contained the same number of bases but no G-rich sequence was used as a negative control (Fig. 1).

**Table 1.** Sequences in random region and dissociation constants of selected DNAs

| DNA clone | Sequence of random region[a] | $K_d$ (M) |
|---|---|---|
| **1**[b] | gagagaGGGGagacaagcgtgtaGGatGGGGaGGGcGGGtGGctgcgattgtttcccca | |
| **2**[b] | actGGGGatGGGtGGGGaGGGctGGGatgcaaattcGGcagtGGaatcgtgtcagactc | |
| **3**[b] | actGGGGatGGGtGGGGaGGGctGGGatgcaaattcGGcagtGGaatcgtgtcagactc | |
| **4**[b] | gaaGGcGGGGGcGGGcacGGGaaGGttGGGttttgcctgtgCCattctcaaGGagcccac | |
| **5**[b] | gagcgtgttGGGtGGGcGGGtgcgaaaGGaGGaGGaGGtccaGGGagatcaccgcgattc | |
| **6**[b] | GGtcGGGcaGGGtGGGtagctGGGaatcGGGGttatgtgtgccagagtGGatgttacttc | |
| **7**[b] | gagcacctGGatcacGGGagtaaacaatagtccgtacacgacaatttgtgatgtcagtcc | n.d.[e] |
| **8**[b] | tatgaaccaaGGGaGGGGcaGGGcGGGtaGGGttaataacGGattccgagcgcccagctc | $1.1 \times 10^{-5}$ |
| **9**[b] | atGGGcGGGataGGGGcGGGGaaGGGattgcGGtgtagtgacgcttGGtgcGGGctatc | |
| **10**[b] | agaatGGttGGcGGtacgaacatcgttGGGaGGaagccgtgatGGGGGaGGGtGGGtac | |
| **11**[b] | aagctagtcacgtgtcgaGGcGGGtGGGGGaGGGcttcctcaGGGacgtGGttacttac | |
| **12**[b] | tcagtctGGcgaatcGGatagcgaGGGGcatgcGGGGtGGGtGGGattGGGtcgcagctc | |
| **13**[b] | acgaagaaacaGGGcgcttcgaacgaacatGGGcaGGGtGGGaagttttaaagtGGtac | $9.1 \times 10^{-6}$ |
| **14**[b] | cagcgtaaGGtcgtGGGtGGttGGGtgcctaagcgtatacttacagcgattgatttGGtc | $1.2 \times 10^{-4}$ |
| **15**[b] | gcgcattaGGGcGGGtatGGGaGGGGtGGactgtatgtcagagcacgcgaGGGGatgtcta | |
| **16**[b] | aatcGGGGttGGGctGGGactGGGtGGGtGGattagagcgcGGttGGaacaGGttgttgc | |
| **17**[b] | GGtgcGGGtaGGGatgaGGGcGGGtcGGGttgagtgtgagcttaGGGGtGGcGGatctac | |
| **18**[b] | GGGcaGGGtGGtgtGGGaGGGGtGGccaGGGtGGaacttgatatcGGGttctaatgcatc | |
| **19**[b] | agctatGGGacGGGattaaatttgccGGGtacgtgtctgtgcatttaatGGGtGGGatgc | |
| **20**[b] | GGtgcGGGtaGGGatgaGGGcGGGtcGGGttgagtgtgagcttaGGGGtGGcGGatctac | $6.3 \times 10^{-6}$ |
| **21**[b] | gaaGGttacGGagtagagagaaaagtgcgccagtgtttttgaGGaGGGaGGGtGGGGagtc | |
| **22**[b] | ctagagtaacctgttGGGaGGGcGGGtaGGGcccattgagaGGaGGacgtattcgtccgc | $1.3 \times 10^{-5}$ |
| **23**[b] | acagctaGGGagccagagtaGGGtGGGGtGGGtatgatgtGGattacttgtatatatgtc | |
| **24**[b] | aGGcGGtaGGGtGGGtGGGttacaccGGGtatagtGGcctGGcatccagcaGGGtgattc | |
| **25**[c] | ccgcacGGaccaGGGtGGGcGGGGtgagaagaaGGcGGGaatactctcgtgcGGtgctagt | |
| **26**[c] | caatGGGGtcGGGcGGGccGGGtgtcatGGtGGacGGagatGGGacgtagaGGGcGGt[d] | $1.6 \times 10^{-6}$ |
| **27**[c] | ccaGGtaactcgctGGtctatttgaaccagccGGGcagaGGGatatGGGaGGGaGGGtatg | |
| **28**[c] | cctcaacGGttGGtgtaaGGGGGcGGGcacaaGGGcacgatcGGGGaacgtaacaatGGgt | |
| **29**[c] | ccgacGGcGGtgaaaGGGattGGGaaGGGtGGGtctgtgagacgtccgatGGtcGGtcttt | |
| **30**[c] | gcGGcaaatcagcatacgagtgtacaGGGacGGGacGGGtGGGtaaaaGGtgtcgcctcag | $2.7 \times 10^{-6}$ |
| **31**[c] | ccaGGGGGagccgatgcGGtgtgagcgaGGaGGGGGtGGtGGGctatacgataccgttttta | |
| **32**[c] | ccaacttGGGcGGaGGGctaacggtGGGGGGatattatgaGGGGtGGaGGtattaaccatt | $9.8 \times 10^{-6}$ |
| **33**[c] | ccGGGGattagaatagtGGaGGGccGGtGGcaaatGGGtaagtaGGttgaaGGGctaaatt | $2.2 \times 10^{-4}$ |
| **34**[c] | ccctGGtccGGtaccaGGGctGGtgcgcaacatttaGGGtGGGtgaGGttGGGatcgttg | |

[a]G-repeated sequences were illustrated by upper case letters.
[b]The DNA clone selected after 7th round.
[c]The DNA clone selected after 10th round.
[d]The underlined sequences were synthesized to determine the binding site. Left sequence was named **26-5′** and right one **26-3′**.
[e]Change of the polarization was not detected.

The clone **26** had two G-rich domains which contained four guanine repeated sequences (Table 1, underlined sequences). To determine which sequence is necessary to recognize HPIX, these two ssDNAs (24-mer, **26-5′**: ATGGGGTCGGGCGGGCCGGGTGTC, **26-3′**: ATGGTGGACGGAGATGGGACGTAG) were synthesized and their binding affinity to HPIX was analyzed (Fig. 2). As a result, **26-5′** (■ in Fig. 2) showed HPIX binding affinity with the $K_d$ value of ca. $1.5\times10^{-5}$ M but **26-3′** (◇ in Fig. 2) had no affinity to HPIX.

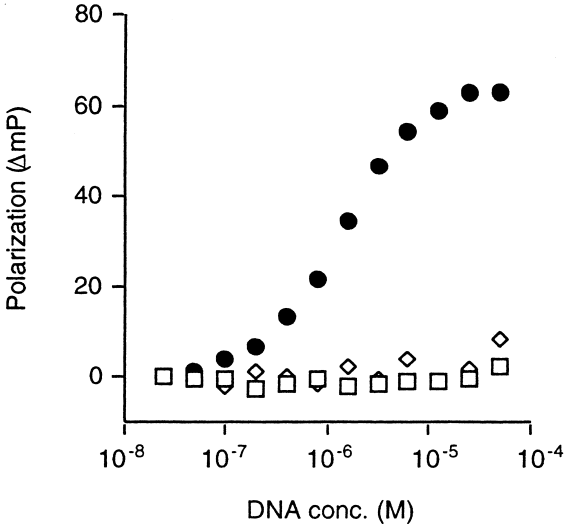


**Figure 1.** Binding curve of the cloned DNAs (**7**: ◇, **26**: ●, negative control: □) with HPIX. The DNAs were diluted sequentially from 50 μM to 0.1 nM, then were mixed with HPIX (1 μM) and incubated for 30 min at room temperature. Polarization was measured using the Beacon 2000 Fluorescence Polarization System (PanVera).

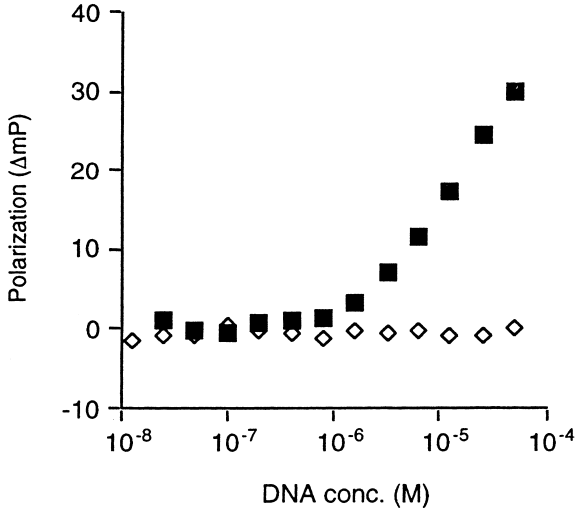


**Figure 2.** Binding curve of the G-rich partial sequences in clone **26** (**26-5′**: ■, **26-3′**: ◇) with HPIX. The DNAs were diluted sequentially from 50 μM to 0.1 nM, then were mixed with HPIX (1 μM) and incubated for 30 min at room temperature. Polarization was measured using the Beacon 2000 Fluorescence Polarization System (PanVera).

## Structural Analysis of HPIX Aptamer

CD spectra of **26-5′** and **26-3′** were recorded using a J-720W (JASCO). The DNA (50 μM in the selection buffer) was heated at 95 °C for 5 min and gradually cooled to 4 °C. Each spectrum corresponds to an average of three scans. The spectrum of **26-5′** had a positive peak at 260 nm and a negative peak at 240 nm (Fig. 3a), which is well consistent with the spectrum of a parallel G-quartet structure (Fig. 4).[9] On the other hand, the

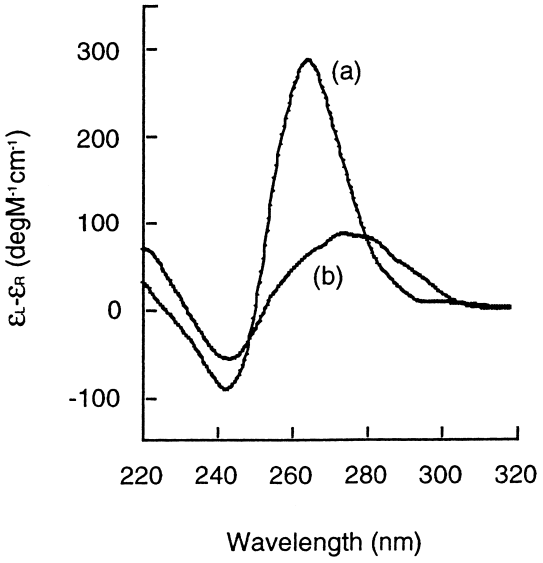


**Figure 3.** CD spectra of (a) **26-5′** (a) and (b) **26-3′**. The DNAs (50 μM in the selection buffer) were heated at 95 °C for 5 min and gradually cooled to 4 °C. Each spectrum corresponds to an average of three scans. CD spectra were recorded using an J-720W (JASCO).

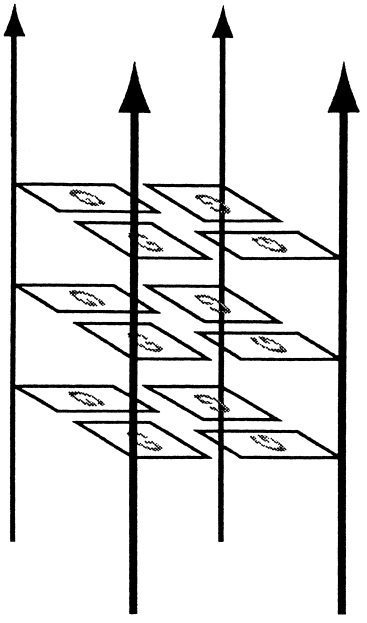

**Figure 4.** Schematic representation of the parallel G-quartet structure.[9]

spectrum of **26-3′** had a relatively broad positive peak around 275 nm (Fig. 3b) showing that the structure of **26-3′** was not as rigid as that of **26-5′**. Each spectrum showed no marked change after HPIX (0.5 mM final concn) was added.

## Discussion

We first tried to determine the $K_d$ values of the aptamers by measuring changes in the fluorescence intensity of HPIX. This method is widely accepted as a way to evaluate the binding affinity between porphyrins and their antibodies[10] or also aptamers,[11] but our aptamers showed no change in fluorescence intensity of HPIX (data not shown). However, the fluorescence polarization analysis clearly indicated that our aptamers bound to HPIX. This result suggested that our aptamers bind to HPIX in a different manner from that of well known interactions between porphyrins and other macromolecules with the increase of fluorescence intensity. It is difficult to analyze fluorescence polarization when the fluorescence intensity of the targeted molecule is changed by binding with their ligands. Thus, our study suggested that the polarization analysis can be adopted to analyze their interaction in such a case that no change in fluorescence intensity is observed.

Our present experiments as well as the previous study[12] suggest that continuous guanine bases in the aptamers may take part in the recognition of porphyrins. To determine the binding site of the aptamer **26**, we measured the binding affinity of two G-rich domains (**26-5′** and **26-3′**) independently. Together with the result of the CD spectroscopic analysis, the domain **26-5′** should take the parallel G-quartet structure (Fig. 4) which is responsible for binding with HPIX. In the past 20 years, the structures of G-rich telomeric DNA sequences have been widely studied. It has been indicated that G-rich sequences form G-quartet structures in vitro.[13] The G-quartets are thought to recognize a variety of ligands such as adenosine/ATP,[14] riboflavin,[15] and so on. However, the structure and the binding mode of those aptamers have not been clearly revealed yet, except the X-ray analysis of thrombine-specific aptamer which was determined to have an exact G-quartet structure.[16] But in contrast to **26-5′**, the thrombine aptamer folds into an anti-parallel G-quartet structure.[16] (CD spectra of ssDNAs folding into anti-parallel G-quartet structures have positive bands at 295 nm.[9]) Some of the G-rich aptamers were shown to act like proteins such as the porphyrin-metalation enzyme[17] or peroxidase.[18] To reveal the relationship between the unique structures and various functions of such telomeric sequences and artificial G-rich aptamers, analysis must be done in both aspects of their structures and dynamics. The structural analysis of our aptamers is currently under study in detail.

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## References

1. Cochran, A. G.; Schultz, P. G. *Science* **1990**, *249*, 781.
2. Nastri, F.; Lombardi, A.; D'Andrea, L. D.; Sanseverino, M.; Maglio, O.; Pavone, V. *Biopolymers* **1998**, *47*, 5.
3. Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505.
4. Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818.
5. Osborne, S. E.; Ellington, A. D. *Chem. Rev.* **1997**, *97*, 349.
6. Patel, D. J. *Curr. Opin. Chem. Biol.* **1997**, *1*, 32.
7. Breaker, R. R. *Chem. Rev.* **1997**, *97*, 371.
8. Dandliker, W. B.; Schapiro, H. C.; Meduski, J. W.; Alonso, R.; Feigen, G. A.; Hamrick, J. R., Jr. *Immunochemistry* **1964**, *1*, 165.
9. Balagurumoorthy, P.; Brahmachari, S. K.; Mohanty, D.; Bansal, M.; Sasisekharan, V. *Nucleic Acids Res.* **1992**, *20*, 4061.
10. Takagi, M.; Kohda, K.; Hamuro, T.; Harada, A.; Yamaguchi, H.; Kamachi, M.; Imanaka, T. *FEBS Lett.* **1995**, *375*, 273.
11. Li, Y.; Sen, D. *Chem. Biol.* **1998**, *5*, 1.
12. Li, Y.; Geyer, C. R.; Sen, D. *Biochemistry* **1996**, *35*, 6911.
13. Wellinger, R. J.; Sen, D. *Eur. J. Cancer* **1997**, *33*, 735.
14. Huizenga, D. E.; Szostak, J. W. *Biochemistry* **1995**, *34*, 656.
15. Lauhon, C. T.; Szostak, J. W. *J. Am. Chem. Soc.* **1995**, *117*, 1246.
16. Padmanabhan, K.; Padmanabhan, K. P.; Ferrara, J. D.; Sadler, J. E.; Tulinsky, A. *J. Biol. Chem.* **1993**, *268*, 17651.
17. Li, Y.; Sen, D. *Nat. Struct. Biol.* **1996**, *3*, 743.
18. Travascio, P.; Li, Y.; Sen, D. *Chem. Biol.* **1998**, *5*, 505.