In Vitro Selection of a DNA Aptamer Binding to Thyroxine

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A DNA aptamer that selectively binds to a hormone, thyroxine, was isolated using the in vitro selection method. A pool of single-stranded DNAs consisting of random sequences was incubated with the thyroxine-immobilized agarose gel; bound DNAs were then collected and amplified by the polymerase chain reaction. This selection process was repeated nine times. The selected DNAs were cloned and sequenced. Some of the cloned DNA contained a consensus sequence. Several DNAs containing the sequence were chemically synthesized and the binding ratios to the thyroxine-immobilized gel were determined. Among them a DNA containing 14 nucleotides was found to bind selectively to thyroxine.

Oligonucleotides that bind with high affinity to their respective target molecules have been discovered by in vitro selection.[1,2] They are called aptamers ('aptus' means 'fit' in Latin). RNA aptamers that bind to various target molecules such as amino acids, bases, nucleotides, cofactors, and proteins have been identified.[3—5] Single-stranded (ss) DNA aptamers that bind to thrombin,[6] organic dyes,[7] and ATP[8] have been isolated using the in vitro selection. We have also used in vitro selection to isolate DNA aptamers for folic acid[9] and methotorexate,[10] and developed a new bioassay system using the aptamers.[11]

Very few studies on the application of aptamers for analytical assay has been reported, although the oligonucleotide aptamers offer several potential advantages over traditional antibody-based reagents, because the aptamers are not derived from living organisms and can be accurately synthesized and reproduced in a short time by automated processes.[12,13] In 1994, Jenison et al.[14] found a RNA aptamer binding theophilline. However, RNA is easily digested by RNAase contained in analytes. Therefore, Drolet et al.[15] reported a modified RNA aptamer, which was nuclease-resistant, for a polypeptide, human vascular endothelial growth factor in 1996. On the other hand, we considered that DNA aptamer is suitable for analytical assay because of the stability and the convenience, and reported pattern-staining by the DNA aptamer in 1996.[9] In the present investigation, DNA aptamers that selectively bind to thyroxine (**1**) were isolated by the in vitro selection (Chart 1); the competitive binding assay of thyroxine was carried out using the aptamers. Thyroxine is a hormone secreted from thyroidea. The molecule is very important for clinical analysis in Japan. The success of analytical assay using DNA aptamer for thyroxine will develop a new field of clinical analysis.

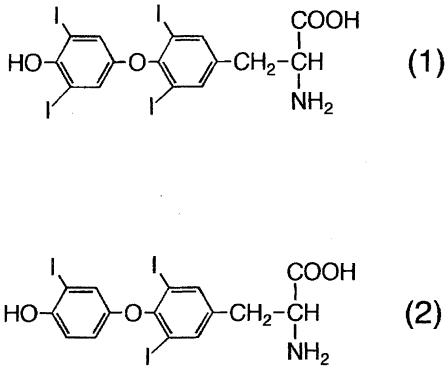

Chart 1.

Materials and Methods

Preparation of Thyroxine- and Lyothyronine-Immobilized Gels. Thyroxine-immobilized agarose gel was purchased from Sigma (St. Louis, MO). Liothyronine (**2**) (Chart 1), which has a structure similar to that of thyroxine, was immobilized on CNBr-activated agarose gel (Pharmacia, Upsala) according to a protocol.[16]

In Vitro Selection. In vitro selection of DNA oligomers specific to thyroxine was carried out as described previously.[9,10] For the first round of selection, synthetic 104-mer oligonucleotides with a random insert of 60 nucleotides, 5′-TAG–GGA–ATT–CGT–CGA–CGG–ATC–C–N_{60}–CTG–CAG–GTC–GAC–GCA–TGC–GCC–G-3′, was amplified using the primers, 5′-TAA–TAC–GAC–TCA–CTA–TAG–GGA–ATT–CGT–CGA–CGG–AT-3′ (P1) and 3′-GTC–CAG–CTG–CGT–ACG–CGG–C-5′ (P2). The synthetic ssDNA (ca. 2μg, ca. 10^9 molecules) was amplified by polymerase chain reaction (PCR) in ten cycles (one cycle: 94 °C, 15 s; 55 °C, 15 s; 72 °C, 15 s) in 100 μL of PCR reaction mixture [2.5 U AmpliTaq® DNA polymerase; 10 mM (1 M = 1 mol dm^{-3}) Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin; 200 μM dNTPs; primers P1 (0.5 μM) and P2 (0.5 μM)]. The ssDNA was then obtained from the amplified double-

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stranded (ds)DNA by additional PCR cycles using only primer P1. The PCR-ssDNA was purified using 3% low-melting agarose gel [NuSieve GTG, FMC BioProducts (Rockland, ME)] in order to remove unreacted primers. The ssDNA pool (ca. 5 μg) was dissolved in 200 μL of binding buffer (0.5 M LiCl; 10 mM Tris-HCl, pH 7.6; 1 mM $MgCl_2$), annealed, loaded onto a column packed with the thyroxine-immobilized gel, incubated for 20 min at 15 °C, and subsequently rinsed with 1.2 mL of the binding buffer (6 column volumes). The bound DNA was then eluted using three column volumes of distilled water. The eluted ssDNA was precipitated with ethanol, rinsed with 70% ethanol, and dissolved in 60 μL of distilled water. One sixth volume of the ssDNA solution was amplified by PCR and used as the input DNA for the next round of selection. This process was then repeated.

The percentage of the DNA binding to the gel was determined by the amount of DNA bound by the column relative to that loaded on the column. The amount of DNA was determined by measuring ultraviolet absorption at 260 nm. UV absorption was measured using a UV/vis spectrophotometer Ubest-50 (JASCO, Tokyo, Japan).

Competitive Binding Assay Using Radioisotope-Labeled DNAs. The selected ssDNAs were labeled with by PCR amplification. The DNAs (5 μg) were amplified by 18 cycles of PCR (one cycle: 94 °C, 15 s; 55 °C, 15 s; 72 °C, 15 s) in the presence of [α-^{32}P]GTP (40 μCi) in 2.0 mL of PCR solution [50 U AmpliTaq® DNA polymerase, 10 mM Tris-HCl, pH 8.3; 50 mM $MgCl_2$: 0.001% gelatin; 200 μM dNTPs; 0.5 μM P1 primer].

The labeled aptamer (400 μL) was bound to thyroxine-immobilized gel (0.8 mL) in buffered solution at room temperature for 20 min. The aptamer-bound gel was rinsed with the binding buffer (10 times volume). A part of the aptamer-bound gel (50 μL) was mixed with a sample solution (200 μL) containing various amounts of thyroxine and liothyronine. After incubation for 20 min at room temperature, the supernatant was collected and the radioactivity was measured.

Cloning and Sequencing. PCR amplified DNA from round 9 was cloned by the T/A cloning method using a kit manufactured by Novagen (Madison, WI), as reported previously.[17] Each clone was sequenced by the dideoxy method, as reported previously.[18]

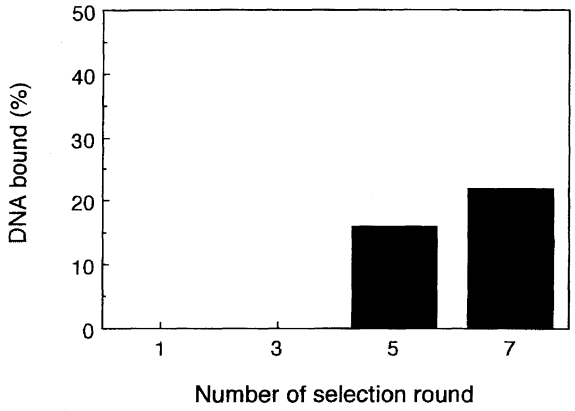

Fig. 1. Binding affinity of selected DNA to the thyroxine immobilized agarose column at each cycle.

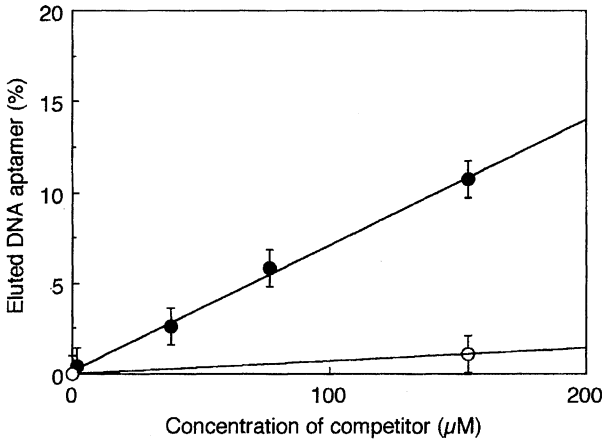

Fig. 2. Elusion of DNA aptamer from the T_4-immobilized gel in the presence of T_4 (●) or T_3 (○). Elusion ratio (%) = radioactivity of supernatant/total radioactivity (supernatant + gel) × 100. The bars represent the standard deviations. $n = 5$.

T4-2: TAATACGACTCACTATAGGGAATTCGTCGACGGATCCGGGCACGCGGAGTAGGGTGGACCAGGAATAGCCCCGGAGCCGTGagcggTCAGGCTTccgctGCAGGTCGACGCATGCGCCG

T4-12: TAATACGACTCACTATAGGGAATTCGTCGACGGATCCGAGGTACGGGGGAGGATGCCCGCCGCAGTatggTCAGGCTTccatGCAAGGAGAGTGTCCTGCAGGTCGACGCATGCGCCG

T4-14: TAATACGACTCACATAGGGAATTCGTCGACGGATCCGGCTGAGTacggTCAGGCTTccgtTGGCGAAGCCGGGGGCCCCAAATCCGATGTGATGTGCTGCAGGTCGACGCATGCGCCG

T4-15: TAATACGACTCACTATAGGGAATTCGTCGACGGATCCATGAATATGGAGATGCTAAGTTGAAGttggTCAGGCCTccaaGGTTAGACTTAGTGCCATCTGCAGGTCGACGCATGCGCCG

T4-17: TAATACGACTCACTATAGGGAATTCGTCGACGGATCCATGAATATGGAGATGCTAAGTCGAAGttggTCAGGCCTccaaGGTTAGACTTAGTGCCATCTGCAGGTCGACGCATGCGCCG

T4-23: TAATACGACTCACTATAGGGAATTCGTCGACGGATCCGCCGTTGGTGTTcggTCAGGCTTccgTGGCAACGGGGCAAAATGGTAATCGCGGGGAACCCTGCAGGTCGACGCATGCGCCG

T4-28: AATACGACTCACTATAGGGAATTCGTCGACGGATCCGAGGTACGGGGGAGGATGCCCGCCGCAGTatggTCAGGCTTccatGCAAGGAGAGTGTCCTGCAGGTCGACGCATGCGCCG

T4-29: AATACGACTCACTATAGGGAATTCGTCGACGGATCCAATCAGtaagcggTCAGGCCTccgcttatCACAGAAAATACGGCGGCGTAGCAGGACAGGCTGCAGGTCGACGCATGCGCCG

Fig. 3. Sequences of cloned DNA aptamers containing a consensus sequence of 12 nucleotides. The consensus sequence motif (boxed) is flanked by several nucleotides (lower case letters) that form a Watson–Crick base-paired stem. Bold type letter indicates minor mutation in the consensus sequence. Constant sequences at the both ends of an aptamer chain are underlined.

NMR Measurement. Chemically synthesized oligonucleotide was purified by ion-exchange chromatography and then gel filtration chromatography, and then was suspended in binding buffer containing 10% D_2O. ^1H NMR spectra were measured at 500 MHz on a Brucker spectrometer.

Results and Discussion

DNAs that bind to thyroxine were isolated by repeated affinity chromatography on thyroxine-agarose column, followed by PCR amplification. The percentages of DNAs binding to the thyroxine column in the selection rounds 1 to 7 are shown in Fig. 1. In the first round of selection less than 1% of the DNAs were bound to the thyroxine column, but after seven rounds of selection and amplification, about 20% of the applied DNAs were bound to the column. A significant rise of the binding ability of DNAs to the immobilized thyroxine was observed as the selection and the amplification cycle repeated.

After seven times of cycle repetition, to eliminate DNA species that bind to both liothyronine and thyroxine, the DNA pool was loaded onto the lyothyronine-immobilized column, and flow-through DNAs were collected and amplified by PCR. This process was repeated twice. This process is called "negative selection" and was utilized to enhance the specific recognition. Jenison et al.[14] selected an RNA ligand that binds with high affinity and specificity to theophilline while showing a much lower binding affinity for other chemicals with similar structures by the same method. They reported

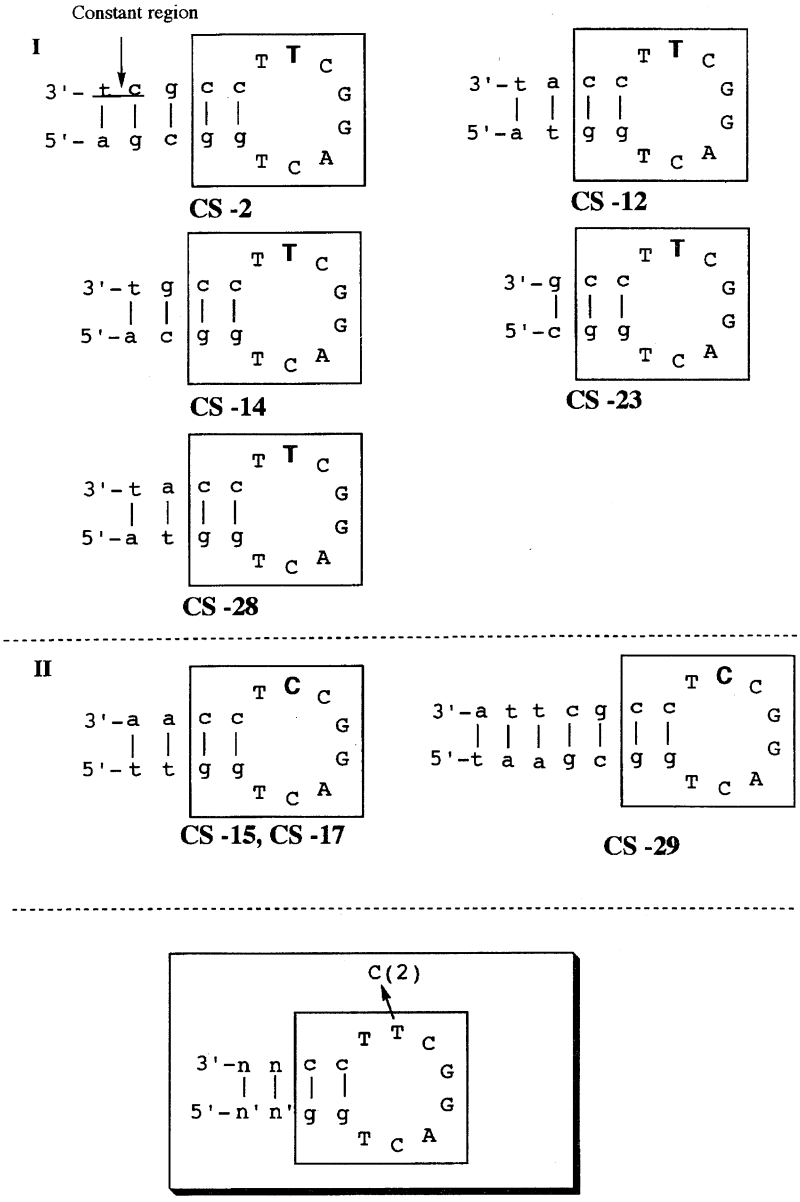

Fig. 4. (a) Secondary structures of DNA aptamers and (b) generalization of aptamers predicted on the basis of the energy calculation. CS-*n* means the consensus sequence of the clone T_4-*n* shown in Fig. 2. The consensus sequence motif (boxed) is flanked by several nucleotides (lower case letters) that form a Watson-Crick base paired stem. Bold type letter indicates minor mutation in the consensus sequence.

that substitution of methyl groups of theophilline altered the dissociation constant from 10 to 10^3 times.

Figure 2 shows the result of competitive binding assay of thyroxine. The DNA aptamers were labeled with ^{32}P-labeled nucleotides and were bound to thyroxine-immobilized gel. Subsequently, the DNAs were eluted from the gel in the presence of various concentrations of soluble thyroxine or soluble liothyronine. DNAs were eluted by the addition of thyroxine, but not by the addition of liothyronine. The amount of eluted DNAs linearly increased with the increase of added thyroxine. DNAs that specifically recognize thyroxine were selected and the system can be used as an assay for thyroxine. About one molecule of DNA was eluted with the addition of ten molecules of thyroxine. The DNA aptamers had apparently 30 μM binding affinity. The binding affinities of DNA aptamers for thrombin, organic dyes, and ATP were reported to be 200 nM, 50 μM, and 6 μM, respectively.[6—8]

Conventional bioassay usually uses polyclonal antibodies for the recognition of target molecules. Similarly noncloned (polyclonal) DNA aptamers will be useful for the assay.

Subsequently, the DNA pool was cloned and 30 clones were sequenced. Among the 30 clones, 8 clones had a consensus sequence composed of 12 nucleotides as shown in Fig. 3, although they consist of two subclasses of consensus sequence, containing C or T. The consensus sequence was found in different distributed sites of cloned DNA. This result indicates that the molecular library was prepared as having random sequences.

Figure 4 shows the secondary structure of consensus sequence estimated by the computer simulation using DNA-folding algorithm.[19] Each sequence has a stem-loop structure. The stem is formed by several base pairs, a part of which are not included in the consensus sequence.

Some truncated DNAs containing the consensus sequence were synthesized by the solid-phase method. The binding affinity to the immobilized thyroxine and liothyronine was examined (Fig. 5). About 15% of DNA (CS-23) composed of the consensus sequence was bound to the thyroxine-immobilized column. Considering that the binding affinity of the full-length clone T4-23 was 17%, the main part participating in molecular recognition was considered to be the consensus sequences. Neither the consensus sequence without stem base pairs (CS-23-LR) nor dsDNA of CS-23 (CS-23-DH) was bound to the thyroxine-immobilized gel. This result indicates the importance of secondary structure of CS-23 in the molecular recognition. In addition, little CS-23 was bound by the liothyronine-immobilized column. The strict molecular recognition by DNA aptamer should be based on the negative selection.

In 1996 Lin and Patel reported the first solution structure of a ligand-DNA aptamer.[20] Their NMR-molecular dynamics structural studies of the interaction between argininamide and a DNA stem-loop complex established that the hairpin loop DNA binding site underwent an adaptive conformational transition on complex formation. The tip of the DNA loop folds down towards the stem and sandwiches the bound argininamide between reversed Hoogsteen A-C and Watson–Crick G-C base pairs. The argininamide is encapsulated within the structured DNA loop and is stablized by an intricate set of intermolecular hydrogen bonds and stacking inter-

Aptamer	Binding ratio (%)	
	T_4	T_3
T4-23	17	—
CS-23	15	3
CS-23-DH	0	—
CS-23-LR	0	—

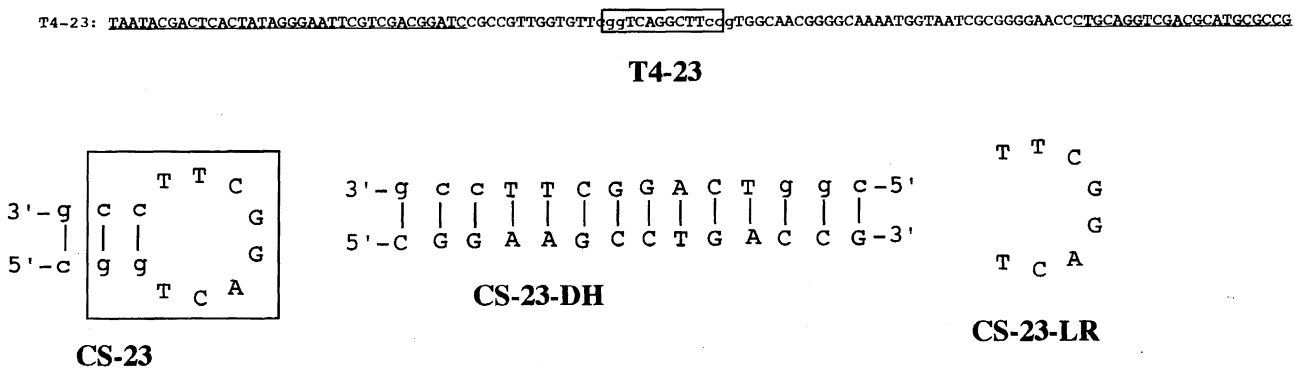

Fig. 5. Binding affinity of chemically synthesized DNAs toward immobilized thyroxine and liothyronine.

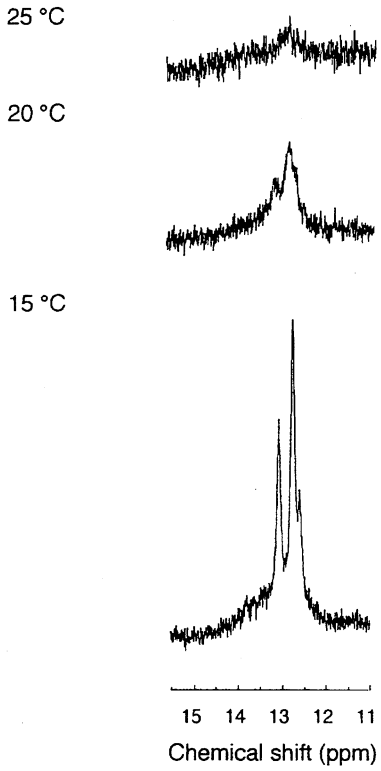

Fig. 6. 500-MHz ^{1}H NMR spectra of the imino resonances of CS-23 at different temperatures.

actions. The solution structure was consistent with the results of chemical footprinting and interference experiments.[21] The structure of the complex demonstrated the molecular principles defining both the architecture of the internal cavity and the recognition elements that could contribute to ligand discrimination.[22]

The structure of CS-23 was investigated by proton NMR spectroscopy (Fig. 6). NMR signals due to the three imino protons of thymine (H3) at 5′ end and guanine (H1) at 3′ end were observed around 13 ppm at 15 °C. Raising the temperature, the intensity decreased and the signal became broad because of an increase of proton exchanges. Such signals indicated three G-C Watson-Crick base pairs between the 5′ and 3′ ends of CS-23. The solution concentration for NMR measurement is so high that it is impossible to distinguish the intramolecular pairing for stem-loop structure from the intermolecular pairing for bulge structure. However, taking into consideration the length of the selected DNA aptamers (120mer) and the concentration of binding solution (μM), we conclude that the intramolecular pairing should be formed in them. In fact, electrophoretic analysis showed that the mobility of DNA aptamers corresponded to that of 120mer ssDNA.

In the present investigation, a DNA aptamer recognizing thyroxine, a clinically important chemical, was obtained. While antibodies are widely used in clinical analysis, the DNA aptamers have advantages over the antibodies in the points of the stability and convenience. The procedures will replace the traditional antibody techniques in the future.

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