Chapter 12
Genetic Alphabet Expansion by Unnatural Base Pair Creation and Its Application to High-Affinity DNA Aptamers

Michiko Kimoto, Ken-ichiro Matsunaga, Yushi T. Redhead, and Ichiro Hirao

Abstract Half a century ago, Alexander Rich proposed a genetic alphabet expansion system by the creation of an artificial extra base pair, known as an unnatural base pair. Now, as an ultimate modification technology, the development of unnatural base pairs and their applications has rapidly advanced. Introducing new components into nucleic acids could increase their functionality and moreover create new types of functional molecules. Three types of unnatural base pairs have been shown to function as a third base pair in replication and transcription. By using the unnatural base pairs, high-affinity DNA aptamers that specifically bind to target proteins and cells have been generated. Furthermore, bacteria bearing an unnatural base pair in their plasmids have been created. Here, we introduce a series of unnatural base pairs that function in replication and transcription, as well as their application to DNA aptamer generation targeting specific proteins.

M. Kimoto
Institute of Bioengineering and Nanotechnology (IBN), 31 Biopolis Way, The Nanos, #04-01, Singapore 138669, Singapore

Center for Life Science Technologies (CLST), RIKEN, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan

PRESTO, JST, Honcho, Kawaguchi-shi, Saitama 332-0012, Japan

K.-i. Matsunaga • I. Hirao (✉)
Institute of Bioengineering and Nanotechnology (IBN), 31 Biopolis Way, The Nanos, #04-01, Singapore 138669, Singapore

Center for Life Science Technologies (CLST), RIKEN, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan
e-mail: ihirao@riken.go.jp

Y.T. Redhead
TagCyx Biotechnologies, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan

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12.1 Introduction

In cellular systems on earth, DNA molecules are replicated and transcribed to RNA by polymerase reactions, according to the fundamental rule of A–T (U) and G–C base pairings. In this biological amplification process, four kinds of nucleoside triphosphates are selectively incorporated into DNA or RNA, opposite each complementary base in DNA templates. Through the amplification process, the information in the original DNA is transferred as base sequences to the offspring DNA or RNA. Furthermore, nucleic acids (DNA and RNA) also act as functionalized molecules, such as enzymes and ligands, and thus their functionalities can be evolved within an organism naturally or in a test tube artificially, by a certain selection process combined with polymerase amplification. Thus, since the advent of the PCR amplification technique, an evolutionary engineering method has been developed to generate functional nucleic acid molecules, such as ribozymes and ligands (aptamers), from an oligonucleotide library with a randomized sequence [1–3]. The method, called in vitro selection or SELEX (Systematic Evolution of Ligands by EXponential enrichment), involves repetitive rounds of selection and amplification to enrich winning nucleic acid sequences in a library. So far, many functional oligonucleotides have been generated by this method. In particular, nucleic acid aptamers that specifically bind to target molecules show promise for diagnostics and therapeutics as next-generation antibodies [4].

However, in the last quarter century since the first SELEX reports, only one modified RNA aptamer (pegaptanib sodium, Macugen) that binds to vascular endothelial growth factor 165 (VEGF165) was approved as a treatment for age-related macular degeneration [5]. The Kd value of the anti-VEGF165 aptamer is relatively high (49–130 pM) [6], but in general, the Kd values of most conventional nucleic acid aptamers are lower (around nM orders), relative to those of antibodies. Although the affinity is not the only problem with nucleic acid aptamers [7], generating tightly binding aptamers to target molecules is the first barrier to overcome for the screening toward diagnostic and therapeutic applications. Thus, increasing nucleic acid functionality, such as aptamer affinity to targets, is an imperative challenge for widespread application of the evolutionary engineering technology.

The restricted functionality of nucleic acids is due to the use of only four kinds of nucleotide components with four bases, as compared to those of proteins, which are composed of 20 standard amino acids. As for aptamers, the high hydrophilicity of nucleic acids is a disadvantageous trait for binding to hydrophobic regions of proteins. Even the heterocycles of the nucleobases are relatively hydrophilic, due to the additional amino and/or keto groups. In accordance with this, the four kinds of nucleotides share very similar chemical and physical properties, thus reducing the diversity of nucleic acid functionality. To compensate for the limited characteristics of the components, in living organisms, various types of nucleotide modifications, especially focusing on the base moiety, exist in functional RNA molecules. In this respect, there have been many reports on artificial

oligonucleotides with modified base, sugar, or phosphate moieties, to improve nucleic acid functionality [8–12]. A successful example is slow off-rate modified DNA aptamers (SOMAmer), in which the 5-methyl groups of thymine residues are replaced with more hydrophobic groups, such as benzyl, naphthyl, or tryptamino groups. This method dramatically increased the success rate of aptamer generation by SELEX, and more than 1000 SOMAmers targeting human proteins were isolated [10, 13]. While the affinities of SOMAmers have also been improved, the Kd values of most nucleic acid aptamers are still on the average of nM orders. The strategies of these modification methods are eventually restricted by the A–T (U) and G–C base pair rules, and thus the number of components consisting of modified oligonucleotides is still limited to four.

Another strategy to improve evolutionary engineering methods is by the genetic alphabet expansion of DNA, using artificial extra base pairs (unnatural base pairs) [14–18] (Figs. 12.1 and 12.2). The creation of an unnatural base pair, functioning as a third base pair together with the natural A–T (U) and G–C base pairs in replication and/or transcription, enables not only an expansion of the genetic information density of nucleic acids but also an increase in the chemical and physical diversities of nucleic acids [19] (Figs. 12.1 and 12.2). If the created unnatural bases have different properties from those of the natural bases, then the chemical and physical diversities of oligonucleotides will be increased. Even though the creation of unnatural base pairs that can be practically used for polymerase reactions had long been a challenging task, unnatural base pairs with high fidelity in replication have recently been developed.

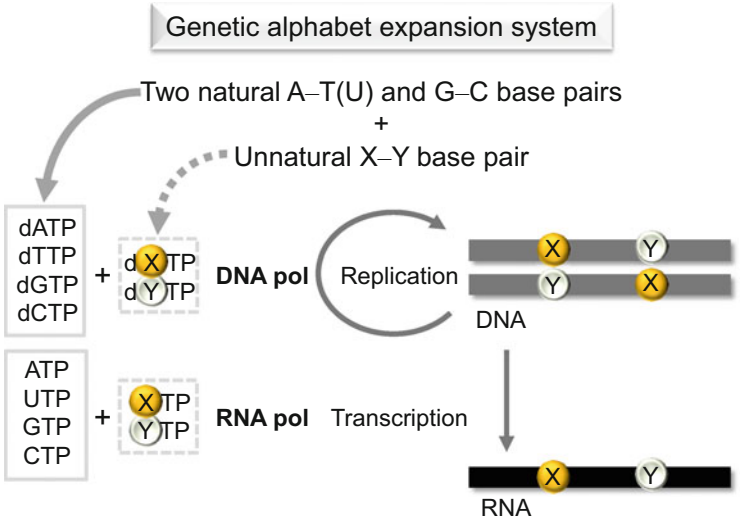

Fig. 12.1 Genetic alphabet expansion system based on the two natural base pairs and an additional, artificial third base pair (unnatural base pair, X–Y) that functions in replication and transcription. This system enables the site-specific incorporation of unnatural components into nucleic acids (DNA and RNA) through polymerase reactions mediated by unnatural base pairs

In 1989, the first experimental demonstration of unnatural base pairs was reported by Benner and co-workers [20, 21]. One of them was the iG–iC pair (Fig. 12.2b), which was proposed by Alexander Rich in 1962 [19]. Unfortunately, the fidelity of the iG–iC pair in replication is not high, because of the tautomerization of the iG base, and most of the unnatural base pair in the DNA is replaced with the A–T pair during PCR amplification. Subsequently, through extensive and consecutive efforts in many proof-of-concept experiments involving the design and physical and biological tests of different types of unnatural base pair candidates, three research groups have created unnatural base pairs with high fidelities in polymerase reactions: (1) our group for the Ds–Pa and Ds–Px pairs (Fig. 12.2c) and others [22–28] (23 is not Ds-Pa and Ds-Px papers), (2) Romesberg's group for the 5SCIS–MMO2 and 5SCIS–NaM pairs (Fig. 12.2d) [29–32] and others [33–35], and (3) Benner's group for the Z–P pair [36–38] (Fig. 12.2e). Recently, genetic alphabet expansion using these unnatural base pairs has been applied to

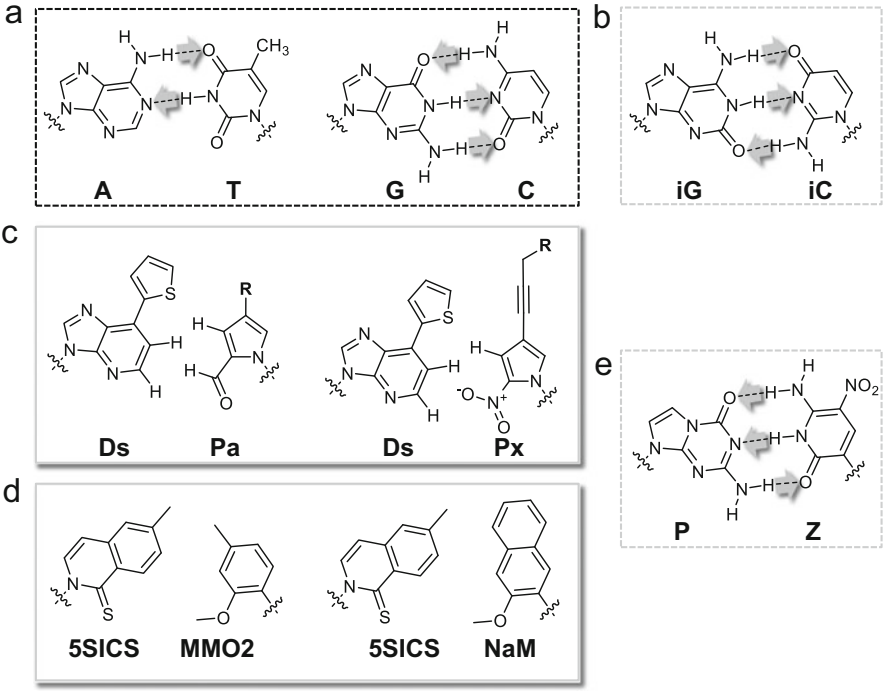

Fig. 12.2 Chemical structures of the natural and unnatural base pairs that function in polymerase reactions. The pairs with hydrogen bonding interactions between the pairing bases are enclosed in dotted lines, and those without hydrogen bonding interactions are enclosed in *solid lines*. The direction from hydrogen bond donor to acceptor is shown with *bold arrows*. (**a**) Natural A–T and G–C base pairs. (**b**) Unnatural iG–iC base pair. (**c**) Unnatural Ds–Pa and Ds–Px base pairs developed by Hirao's group. (**d**) Unnatural 5SICS–MMO2 and 5SICS–NaM base pairs developed by Romesberg's group. (**e**) Unnatural P–Z base pair developed by Benner's group. R is a functional group

generate high-affinity DNA aptamers targeting proteins by SELEX [39] and a cancer cell line by Cell-SELEX [40] and to create engineered *Escherichia coli* capable of replicating plasmid DNA containing an unnatural base pair [41].

Here, we provide an overview of the creation of unnatural base pairs by the three research groups and their applications. In Sect. 12.2, we introduce a series of unnatural base pair studies by the three independent groups, which finally succeeded in the development of unnatural base pairs that function in replication and transcription as a third base pair. Among them, the application of the hydrophobic Ds–Px pair to SELEX procedures is highlighted in Sect. 12.3. We describe the process of generating a high-affinity DNA aptamer composed of five nucleotides (A, G, C, T, and Ds) targeting VEGF165 and interferon-γ (IFNγ), including the process of the aptamer species enrichment during the selection.

12.2 Creation of Unnatural Base Pairs

12.2.1 Hirao's Base Pairs

The main design concept of our unnatural base pairs, such as Ds–Pa and Ds–Px, is to strictly refine the shape-complementarity between pairing bases, in which the ideas of steric hindrance and electrostatic repulsion are also included, to avoid mispairings (non-cognate pairings) between unnatural and natural bases. The initial idea of the shape-complementarity of pairing bases in replication was developed by Kool's group, using natural base analogues [42, 43]. We expanded this idea and developed a series of hydrogen bonded and hydrophobic unnatural base pairs that function in replication, transcription, and/or translation as a third pair [16, 17, 44].

The first generation of our unnatural base pairs includes the hydrogen-bonded pairs between a large base analogue, such as 2-amino-6-(*N*,*N*-dimethylamino) purine (x) [45, 46], 2-amino-6-(2-thienyl)purine (s) [23, 47], and 2-amino-6-(2-thiazoyl)purine (v) [48–50], and a small base analogue, such as pyridin-2-one (y) and imidazolin-2-one (z) [51] (Fig. 12.3). The x–y, s–y, and v–y pairs have two hydrogen bonds, but their donor–acceptor geometries are different from those of the natural A–T(U) and G–C pairs, like those in the iG–iC pair. Furthermore, to remove the mispairing with the natural bases, these large base analogues have sterically hindered groups at position 6. Among them, the s–y pair is useful for the site-specific incorporation of y or modified y bases into RNA, opposite s in DNA templates, by transcription using T7 RNA polymerase. The y base can be linked to desired functional groups, such as biotin and fluorescent groups, as a side chain, and these modified y substrates can also be incorporated into RNA by T7 transcription [48, 50, 52, 53].

In contrast to the y base with the six-membered ring, the five-membered z base increases the steric fitting with the s and v bases and decreases that with A. The s–z and v–z pairs are useful for the site-specific incorporation of s and v into RNA,

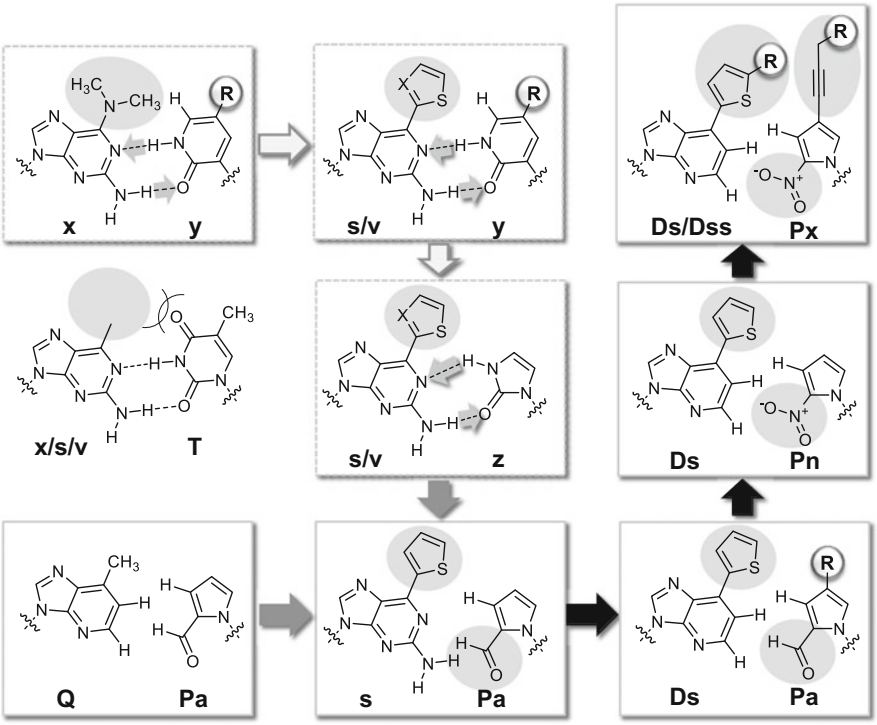

Fig. 12.3 Hirao's unnatural base pair development process: from the x–y, s/v–y, and s/v–z pairs as the first generation, to the s–Pa pair derived from the first generation and the Q–Pa pair as the second generation, followed by the hydrophobic Ds–Pa, Ds–Pn, and Ds/Dss–Px pairs as the third generation. Undesired, non-cognate pairing between the x/s/v–T pair is also shown as an example. The R enclosed in the circle represents functional groups

opposite z in templates, by T7 transcription [49, 51]. Since the s and v bases are fluorescent, the use of these s–z and v–z bases allows the site-specific fluorescent labeling of RNA molecules.

The second generation of our unnatural base pairs is between hydrophilic and hydrophobic base analogues. The unnatural base pair between s and a relatively hydrophobic base analogue, pyrrole-2-carbaldehyde (Pa), further increased the transcription efficiency and selectivity of the s incorporation into RNA opposite Pa in templates, as compared to the s–z pairing [26, 27] (Fig. 12.3). By T7 transcription using the s–Pa pair, a series of site-specific, fluorescently labeled functional RNA molecules, such as a tRNA and a ribozyme, were synthesized [54]. Since the fluorescent intensity of the s base embedded into RNA is decreased by the stacking with neighboring bases [55], the local structural changes of functional RNA molecules can be analyzed, depending on the environmental conditions [26, 27, 54].

The third generation is hydrophobic unnatural base pairs, such as the Ds–Pa, Ds–Pn, Ds–Px, Dss–Pa, Dss–Pn, and Dss–Px pairs, which function complementarily

and exhibit high fidelity in replication and transcription as third base pairs with different shape-complementarity from those of the natural base pairs [22, 24, 25, 28, 56]. By removing the hydrogen-bonding residues from both of the pairing bases, these hydrophobic base pairs can be practically used for PCR amplification. The shapes of the pairing bases snugly fit each other, and in particular, the oxygen of the nitro group in Px electrostatically clashes with the 1-nitrogen of A, reducing the non-cognate A–Px pairing. Thus, the Ds–Px pair exhibits extremely high fidelity in PCR. In replication using exonuclease-proficient Deep Vent DNA polymerase, the selectivity of the Ds–Px pairing is as high as 99.9 % per replication, and the misincorporation rate of the unnatural base substrates opposite the natural bases in templates is as low as 0.005 % per base pair per replication, corresponding to an error rate of 5×10^{-5} error per base pair. The Px base can be modified, and the selectivity of the Ds and modified Px base pairs in PCR depends on its modifications [25]. The Ds–Px pair has been applied to real-time PCR techniques [57] and SELEX for generating high-affinity Ds-containing DNA aptamers [39], as described in the next section.

The Dss base, with an additional thienyl group (R = thienyl of Ds/Dss in Fig. 12.3), is a highly fluorescent base analogue, and the nitropyrrole moiety of Pn and Px acts as a quencher [28, 58]. Thus, the Dss–Pn and Dss–Px pairs are quite unique third base pairs between a fluorophore and a quencher, and have been applied to real-time quantitative PCR and molecular beacon techniques [28, 54, 57].

12.2.2 Romesberg's Base Pairs

In the late 1990s, Romesberg's group initially reported the hydrophobic self-base pair of propynyl isocarbostyril (PICS, Fig. 12.4) [35]. Subsequently, they designed and synthesized a series of different hydrophobic base analogues, and huge combinations (~1800) of each analogue were tested in replication [59–69]. Among them, they selected the unnatural base pair between SICS and MMO2 as the prototype (Fig. 12.4) [70]. Upon further optimization, they created two hydrophobic pairs, 5SICS–MMO2 and 5SICS–NaM [30,71] (Fig. 12.4). The 5SICS–NaM pair functions in PCR using One*Taq* DNA polymerase (a mixture of DeepVent DNA polymerase and *Taq* DNA polymerase) with ~99.9 % fidelity. The 5SICS–NaM pair also functions in transcription using T7 RNA polymerase [29–32, 71]. Their continuous exploration has yielded further unnatural base pairs, such as the TPT3–NaM pair [33] (Fig. 12.4).

In 2014, by using the two unnatural base pair systems of the 5SICS–NaM and TPT3–NaM pairs, they created the first semi-synthetic living *E. coli*, in which the artificial plasmid DNA containing their unnatural base pair can be precisely amplified when supplemented with the unnatural base triphosphate substrates from the culture media [41] (Fig. 12.4). The TPT3–NaM pair was used for the PCR amplification of a plasmid bearing the unnatural base pair [34], and the 5SICS–NaM pair

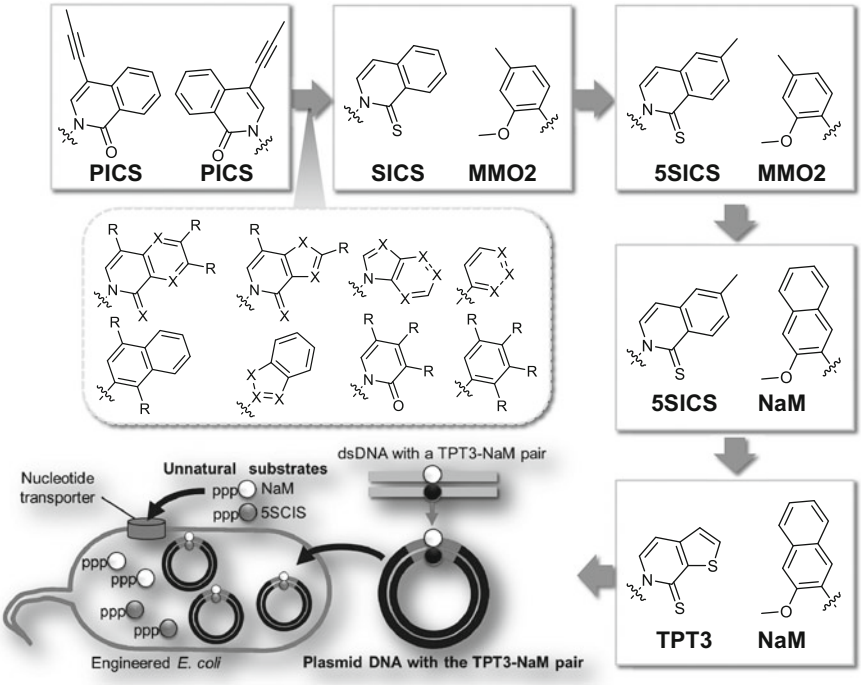

Fig. 12.4 Romesberg's hydrophobic unnatural base pairs and the introduction of the unnatural components into a plasmid, which can be replicated within an engineered *E. coli* strain. Representative nucleobase scaffolds and substitutions (X: heteroatom, R: functional group), enclosed in a *dotted square*, were used for screening of functional unnatural base pairs [70], resulting in the parent SCIS– MMO2 pair

was used for the plasmid-containing *E. coli* propagation. To supply the unnatural base triphosphates to the cells, they employed a triphosphate transporter from a microalgae. *E. coli* was transformed with two plasmids, one encoding the transporter and the other containing one unnatural TPT3–NaM pair. After approximately 24 doublings (a 15-h period of growth) with the 5SCIS and NaM triphosphates supplemented in the culture medium, the transformed *E. coli* cells maintained 86 % of the base pair in the plasmid, indicating that these unnatural nucleosides could survive in the presence of the natural DNA repair systems in the cell.

12.2.3 Benner's Base Pairs

In the late 1980s, Benner's group reported several types of unnatural base pairs with different hydrogen-bond geometries from those of the natural base pairs. One of them is the iG–iC pair (Fig. 12.2b), which was applied to transcription for the amino modification of RNA [72] and to real-time multiplex PCR (Plexor system) [73–

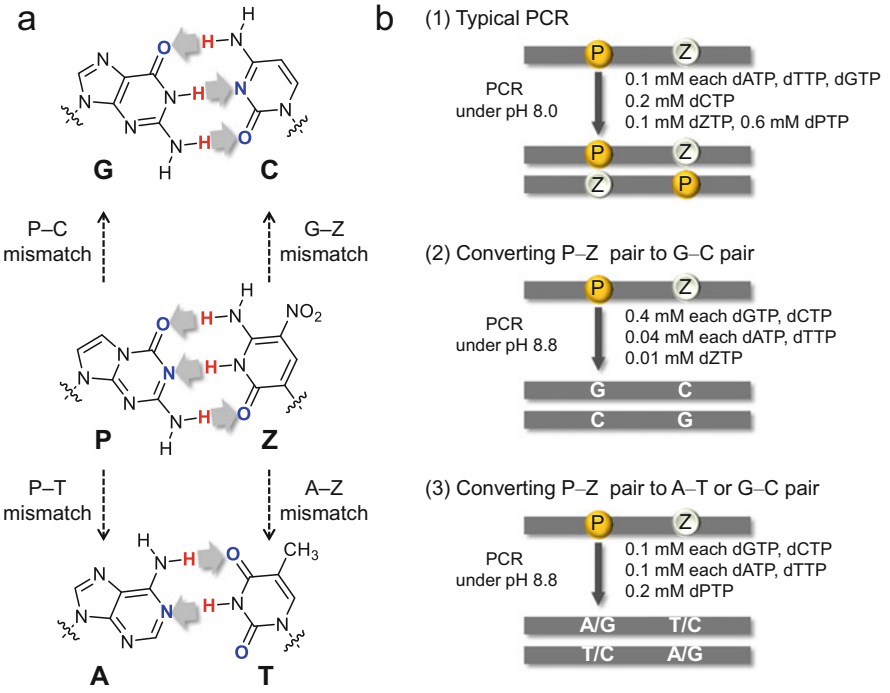

Fig. 12.5 Benner's unnatural P–Z base pair, with a different hydrogen bonding pattern from those of the natural base pairs. (**a**) Conversion of the cognate P–Z base pair to the natural G–C and A–T base pairs via possible mismatch pairing. (**b**) PCR conditions used in Benner's Cell-SELEX, for typical replication to prepare the DNA library (1), and for replacing the unnatural P–Z pairs with the natural G–C base pairs (2) or with the natural G–C and A–T base pairs (3) for sequence determination. Comparison of the replacement patterns in (2) and (3) allows the identification of the original P or Z positions in DNA

75]. They named their unnatural base pair system Artificially Expanded Genetic Information Systems (AEGIS) [20, 21, 38, 76]. To adapt AEGIS to a high-fidelity replication system, they examined several types of unnatural hydrogen-bonded base pairs [76–81]. In 2006, they created an unnatural base pair between 2-aminoimidazo[1,2-*a*]-1,3,5-triazin-4(8*H*)-one (P) and 6-amino-5-nitro-2(1*H*)-pyridone (Z) (Figs. 12.2e and 12.5a) [38], which can be used in PCR with 99.8 % fidelity [37] using *Taq* DNA polymerase and applied it to several DNA-based biotechnologies [40, 82, 83].

In 2014, they reported the generation of DNA aptamers that bind to a breast cancer cell line (MDA-MB-231) by a Cell-SELEX method involving the P–Z pair, in collaboration with Tan's group [40]. The initial DNA library for Cell-SELEX was prepared by the chemical synthesis of 52-mer DNA fragments bearing a 20-base randomized region consisting of six different bases. After 12 selection rounds, the unnatural bases in the enriched library were replaced with the natural bases by an additional PCR amplification using two different PCR conditions in the

absence of the unnatural base substrates, as shown in Fig. 12.5a, b. Since the P–Z pair was forcefully converted to the G–C pair or to the mixture of the G–C and A–T pairs [37, 40] depending on the PCR conditions, the possible P or Z positions in the randomized region can be identified by comparing the two sequence data sets. Consequently, they obtained a DNA aptamer containing both P and Z that binds to MDA-MB-231 cells with a Kd value of 30 nM.

12.3 Example of Unnatural Base DNA Aptamer Generation

As an example of applications using genetic alphabet expansion, here we describe high-affinity DNA aptamer generation using our Ds–Px pair system. Since nucleic acid aptamers are generated by an evolutionary engineering method (SELEX) composed of repeated cycles of selection and PCR amplification [1, 3], SELEX is an attractive and germane demonstration to test the ability of the replicable unnatural base pairs. We developed a genetic alphabet expansion SELEX method and generated a couple of DNA aptamers that specifically bind to target proteins, such as VEGF165 and interferon-γ, with >100-fold higher affinity than conventional DNA aptamers containing only natural bases [39]. Several ideas were adopted to improve the new SELEX method, and then we learned how to conduct the evolutionary process to isolate high-affinity DNA aptamers, via firsthand experience by performing the SELEX procedure using a randomized DNA library consisting of five different bases.

The key to the new SELEX procedure is the DNA library containing unnatural bases. We chose Ds as the fifth base and did not add Px to the library. The high hydrophobicity of the Ds base enhances the interactions with hydrophobic regions of target proteins. In addition, the absence of the pairing partner of Ds increases the structural diversity of each oligonucleotide in the library and causes the protrusion of the Ds base from the aptamer scaffolds, facilitating the interactions between the Ds base and the hydrophobic cavities of target proteins. From this perspective, we presumed that only a few Ds bases would be required to affect protein binding, and thus one to three Ds bases were introduced within a 43-base randomized region in a DNA library. This single-stranded Ds-containing DNA library can be amplified by PCR in the presence of the four natural and two unnatural base substrates by AccuPrime Pfx polymerase [25]. By PCR amplification using a primer linked with an extra tag, the Ds strands were separated from the Px strands with an extra tag on a denaturing gel, for the following selection cycles.

Currently, a major barrier is how to determine each sequence containing Ds bases from the isolated library after the SELEX procedure. In general, when using natural-base libraries, classical cloning and sequencing methods or a next-generation sequencing method can be used for DNA library sequencing. However, these conventional methods cannot be directly applied to the sequencing of the

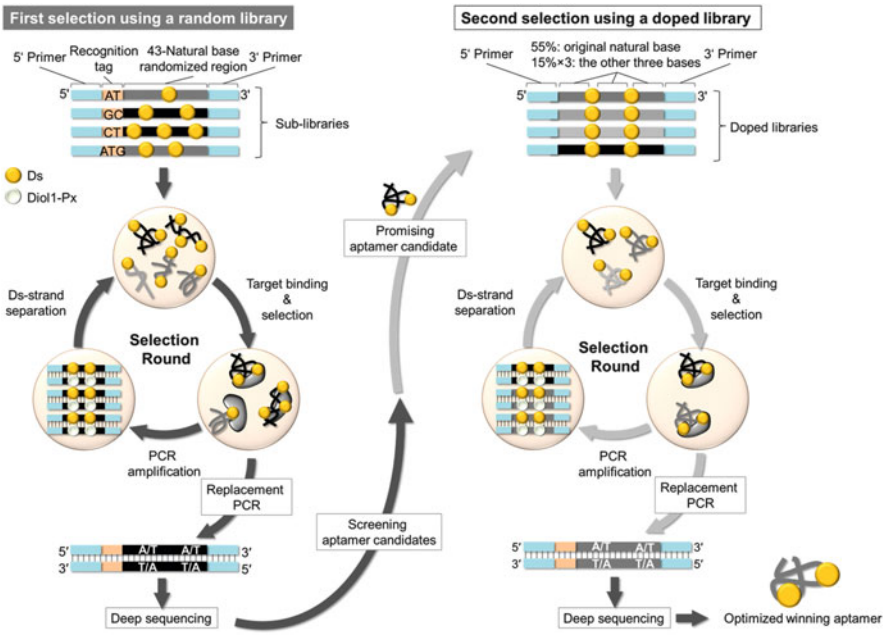

Fig. 12.6 Scheme of the SELEX procedure by Hirao's group for DNA aptamer selection using libraries containing hydrophobic Ds bases [39]. In the first selection, a mixture of 22 different chemically synthesized sub-libraries was employed as the initial library. In each selection round, target-binding DNA fragments were extracted and then amplified by PCR in the presence of dDsTP and dPxTP as unnatural substrates, together with natural dNTPs for the next round of selection. The enriched DNA library was PCR-amplified without dDsTP and dPxTP, resulting in the amplicons consisting of only natural bases, followed by sequence analysis. Details of the "replacement PCR" are described in the main text (see Sect. 12.3). After binding analyses of the obtained aptamer candidates, the most potent aptamers proceed to the second selection using a doped library. Through the doped selection and further optimization, the final "winning" aptamers were obtained

library containing unnatural bases, at present. To address this problem, we created a simple, but effective, approach using a set of Ds-containing DNA sublibraries. The sublibrary construction and the SELEX method are summarized in Fig. 12.6.

Twenty-two different sublibraries, in which one to three Ds bases were embedded at specific positions of a randomized 43-natural-base region, were chemically synthesized. Each sublibrary contained one to three Ds bases at different positions and also had a specific barcode consisting of two or three natural base sequences, as a recognition tag. By using the mixture of 22 sublibraries, we performed seven rounds of selection and amplification for VEGF165 and IFNγ. After the selection, the isolated library was again amplified by PCR in the absence of the Ds and Px substrates, but in the presence of the Pa substrates. By this process, the Ds–Px pairs in the library were efficiently replaced with the natural base pairs, mainly A–T pairs. Due to the high fidelity of the Ds–Px pair, the replacement was very difficult to conduct in PCR with only the natural base substrates, and thus we added another

unnatural base substrate, dPaTP. First, the Pa substrate was incorporated into the complementary strand opposite Ds in the library, and then the A substrate was incorporated opposite Pa, promoting the replacement of the Ds–Px pair with the A–T pair by PCR. The PCR products were sequenced by Ion Torrent deep sequencing. Using the barcode in each sequence, we could identify the initial Ds base positions. From the appearance frequency of each clone, several sequences with a high frequency were chosen from more than 90,000 sequences and were assayed for their ability to bind the target proteins. Among them, the aptamer sequence of the strongest binder was optimized by a second SELEX, using a doped library with a partially mutated sequence.

Table 12.1 shows the in vitro selection conditions used for the anti-IFNγ aptamer generation [39]. The selection pressure was gradually increased in each selection round, by reducing the concentrations of the DNA library and IFNγ, adding a competitor (the conventional DNA aptamer) [84, 85], and/or harsh washing with 3 M urea. We monitored the enrichment of the aptamer species during the selection by surface plasmon resonance (SPR) and gel-mobility shift assays of the library in each round (Fig. 12.7). After four rounds in the first SELEX, the binding species were significantly enriched (Fig. 12.7a, b). From SPR, the sequences were continuously enriched from the fourth to seventh rounds while the binding affinity increased. In the second round of SELEX, using the doped library, the binding species appeared quickly after two rounds (Fig. 12.7c).

Table 12.2 shows the theoretical copy numbers in the initial doped library used in the experiments targeting IFNγ and summarizes the data obtained after four rounds of the doped selection. The theoretical ratio of the optimized sequence in the initial library population; i.e., the number for the finally obtained optimized sequence, with one mutation at a specific position divided by that of the original

Table 12.1 Conditions of SELEX involving the Ds–Px pair, targeting human IFN-γ[a]

DNA library	Selection round	DNA		IFN-γ (nM)	Competitor (nM)	Number of washes		PCR cycles
		Pmol	(nM)			Without urea	With 3 M urea	
Random	1	300	50	25	0	5	0	22
	2	25	25	10	0	5	0	21
	3	5	5	5	0	5	0	21
	4	3	1	1	0	5	0	26
	5	3	1	1	100	5	0	20
	6	3	1	1	500	5	0	20
	7	3	1	1	500	2	3	25
Doped	1	300	50	25	0	5	0	19
	2	5	5	5	0	5	0	20
	3	3	1	1	100	5	0	17
	4	3	1	1	500	2	3	20

[a]The conditions for the first selection using a DNA library containing Ds at predetermined positions and the second selection using a doped DNA library containing three Ds bases at fixed positions of the original sequence are summarized [39]

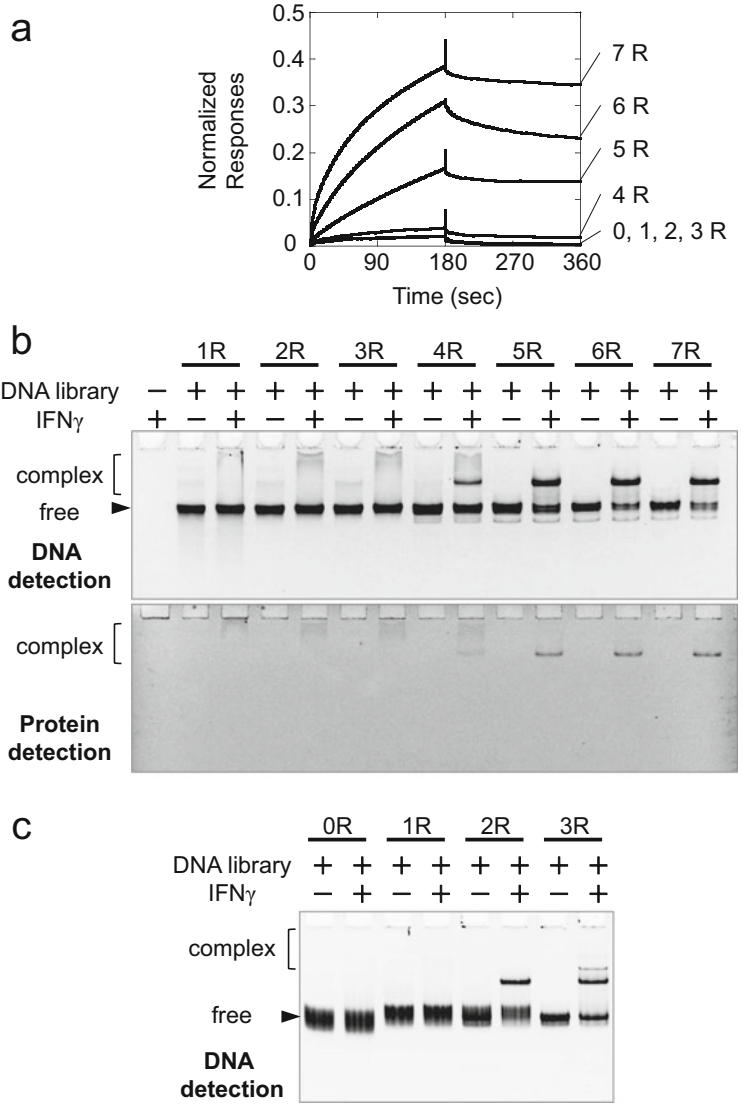

Fig. 12.7 Monitoring the enrichment process of the DNA libraries in SELEX targeting IFNγ. The conditions used in each selection round are summarized in Table 12.1. (**a**) SPR analysis of DNA libraries after each selection round in the first SELEX procedure. Each DNA library was immobilized via hybridization on a streptavidin-coated sensor chip with a biotinylated DNA probe. SPR conditions: flow rate = 30 μl/min; running buffer: PBS (305 mM NaCl) supplemented with 0.05 % Nonidet P-40, pH 7.4; injection period of 100 nM IFNγ = 180 s, dissociation period for monitoring = 180 s. (**b**) Gel-mobility shift analysis of DNA libraries after each selection round in the first SELEX procedure. Binding conditions: 100 nM DNA library, human IFNγ 200 nM, incubation at 25 °C for 30 min in PBS (155 mM NaCl) supplemented with 0.005 % Nonidet P-40. Gel electrophoresis: 10 % polyacrylamide gel containing 5 % glycerol, with 0.5× TBE as the running buffer. DNA bands were detected by SYBR Gold staining (*upper panel*), and protein bands were detected by CBB staining (*lower panel*). (**c**) Gel-mobility shift analysis of DNA libraries after each selection round in the second doped SELEX. Binding conditions: the same

Table 12.2 Ratios of enriched sequences after four rounds of the doped selection targeting human IFNγ[a]

Mutations relative to the original aptamer candidate sequence (N)	Variations of different sequences (X)[b]	Theoretical copy numbers in the initial doped library (1.8×10^{14} molecules)	After 4 rounds of the doped selection: Total extracted reads: 73,918		
			Reads	Variations	Ratio of the total reads (%)
0	1	2241	55	1	0.07
1	126	611	29,791 (29,518)[d]	3	40.30 (39.93)[d]
2	7749	167	18,980	24	25.68
3	309,960	45	10,819	57	14.64
4	9,066,330	12	4976	84	6.73
5	206,712,324	3	1776	75	2.40
6	3,824,177,994	1	1518	147	2.05

[a]Each doped position (42 sites) in the initial doped library was 55 % original and 15 % each different natural base, and 1.8×10^{14} molecules (300 pmol) were used as the initial library for the second doped selection [39]
[b]X is calculated according to the formula: $3^N \times {}_{42}C_N$
[c]The number is calculated according to the formula: $(1.8 \times 10^{14}) \times 0.55^{(42-N)} \times 0.45^N \times {}_{42}C_N/X$
[d]The top ranked, optimized sequence after the 4th round of the doped selection

sequence, is 0.27 (611/2241), and then the value reached 536.69 (29,518/55) after the doped selection, resulting in an almost 2000-fold enrichment. Doped selections allow the identification of the optimized sequence, which cannot be covered in the first selection using a random library, and also provide useful sequence information to shorten the length of the original aptamer candidate and to estimate the important secondary structure for target binding [39, 86].

Finally, we obtained an anti-VEGF165 aptamer with a 47-mer containing two Ds bases and an anti-IFNγ aptamer with a 49-mer containing three Ds bases. The secondary structures of both aptamers (Fig. 12.8) were presumed from the sequence data obtained by the second SELEX, in which the stem regions could be assigned from the co-variation mutation forming base pairs [86]. The Kd values, determined by SPR, were extremely high, 0.65 pM for the anti-VEGF165 aptamer and 38 pM for the anti-IFNγ aptamer. These binding affinities greatly surpass those of the conventional DNA aptamers consisting of only natural bases [84, 85], as well as the Ds→A mutants of the Ds aptamers, by more than 100-fold. We also confirmed that each unnatural base DNA aptamer specifically binds only to its target protein. Based on the mutant analysis, two of the three Ds bases in the anti-IFNγ aptamer

Fig. 12.7 (continued) as in (**b**) except human IFNγ 100 nM. Gel electrophoresis: the same as in (**b**) except 8 % polyacrylamide gel containing 5 % glycerol, with 0.5× TBE as the running buffer

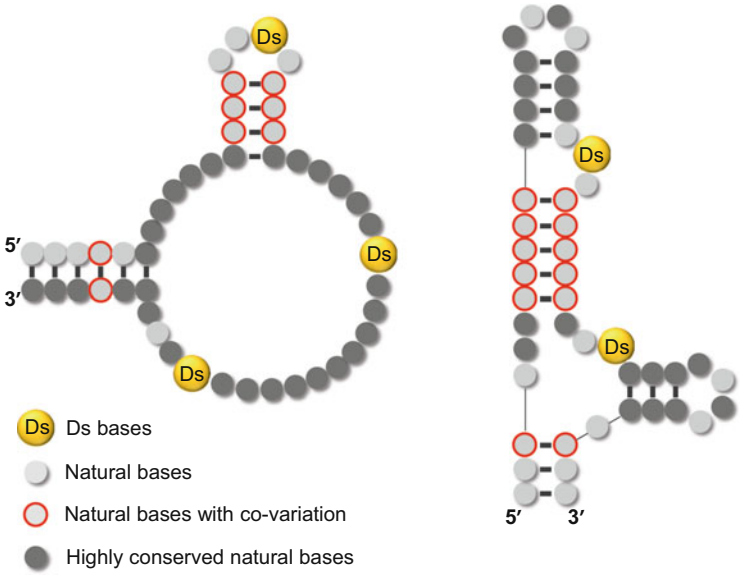

Fig. 12.8 Predicted secondary structures of the anti-IFNγ aptamer (*left*, 49-mer) and the anti-VEGF165 aptamer (*right*, 47-mer). The sequence alignment information obtained from the second doped selection is mapped on each structure. *Thick-framed circles* indicate positions where co-variations were found to form a base pair, and *dark solid circles* indicate highly conserved bases (the conservation percentage of each base in the total clone number was more than 96 %) [39]

were essential, and, thus, only two Ds bases greatly affected the tight binding to the target proteins, IFNγ and VEGF165.

12.4 Conclusion

Here, we have introduced three types of unnatural base pairs that can be used for PCR amplification. DNA aptamer generation using genetic alphabet expansion is the first demonstration to prove the importance of the introduction of new components for creating biopolymers with increased functionality. Since a bacterial strain bearing plasmids containing an unnatural base pair has been reported [41], novel bacteria producing useful pharmaceutical products or materials related to energy production could be created. Eventually, genetic alphabet expansion could be applied to the production of new peptides and proteins containing nonstandard amino acids, which would be useful for producing antibody–drug conjugates. Before that, unnatural-base DNA aptamers might contribute to the further advancement of nucleic acid pharmaceutics.

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