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Influence of DNA Aptamer Structure on the Specificity of Binding to *Taq* DNA Polymerase

O. Yu. Yakimovich[1]*, Ya. I. Alekseev[2], A. V. Maksimenko[1], O. L. Voronina[3], and V. G. Lunin[1]

[1]All-Russia Institute of Agricultural Biotechnology, ul. Timiryazevskaya 42, Moscow, 127550 Russia; fax: (095) 977-7455; E-mail: yakimovich@mk.ru
[2]JSC Syntol, ul. Timiryazevskaya 42, Moscow, 127550 Russia
[3]Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow, 117871 Russia

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Abstract—The secondary structure of DNA aptamer to *Taq* DNA polymerase was established as a hairpin. Both stem and loop structures of DNA ligand were shown to be involved in the interaction with *Taq* DNA polymerase. Moreover, the structure and sequence of DNA aptamer that was the most effective inhibitor of DNA polymerase activity were established. This crucial structure was evaluated as a GC-rich stem longer than 17 bp, and a loop consisting of 12 bases with strictly determined nucleotide sequence. It was demonstrated that nucleotide in position 23 counting from the 5′-end of DNA ligand was involved in direct contact with *Taq* DNA polymerase. The ability of optimized DNA aptamer TQ21-11 to form a complex with the enzyme was increased 5-fold in comparison to the initial aptamer.

Key words: DNA ligands, DNA aptamer design, *Taq* DNA polymerase, DNA–protein complex, PCR

Taq DNA polymerase is a thermostable and thermoactive (optimal activity occurs at 75-80°C) enzyme of the bacterium *Thermus aquaticus*. It is a representative of DNA dependent DNA polymerases I that are involved in DNA replication and reparation in prokaryotic organisms [1]. *Taq* DNA polymerase consists of one subunit of 832 amino acids with molecular weight 93,920 daltons. Two domains are distinguished within the enzyme: C-terminal catalyzing DNA polymerization, and N-terminal accomplishing 5′-3′-exonuclease DNA hydrolysis.

Actually, both the mechanism of *Taq* DNA polymerase action and the structure of the domains responsible for DNA polymerase and 5′-3′-exonuclease activities have been intensively studied [2-11].

New possibilities for the investigation of the structural peculiarities of biological molecules were opened by the method of *in vitro* selection SELEX (Systematic Evolution of Ligands by Exponential enrichment) developed in 1990. This method provides the possibility to construct nucleotide ligands—aptamers (Latin *aptus*, suitable)—of small size and unique three-dimensional structure. Aptamers are able to interact with various target molecules with high specificity and affinity [12, 13].

Two aspects define the interest in the structural study of aptamers to DNA- and RNA-binding proteins. First, knowledge of the structure of DNA- or RNA-ligand permits investigation of the organization of the enzyme–substrate (inhibitor) complex without application of complicated and expensive methods.

Second, activating or inhibiting capacities of aptamers can be improved by changing the structure of DNA ligand, thus providing the possibility to design effective inhibitors or activators of enzymes [14-18].

DNA aptamers to *Taq* DNA polymerase were designed in 1996 [19]. The aptamers were able to bind *Taq* DNA polymerase at room temperature (25-35°C) and did not interact with the enzyme at temperatures higher than 40°C.

DNA ligands were classified in two families according to their homologies. One DNA ligand with maximal affinity to the enzyme was specified within each family (TQ30 and TQ21, respectively). It has been suggested that the variable part of the aptamer TQ30 was a hairpin [20]. The secondary structure of TQ21 aptamer has not been discussed by the authors, but it has been shown that a fragment of this DNA ligand consisting of 51 nucleotides was involved in the interaction with the enzyme [20].

Abbreviations: PCR) polymerase chain reaction; dNTP) nucleoside-5′-triphosphate; K_d) dissociation constant; IT_{50}) temperature of 50% polymerization of model oligonucleotide in the presence of aptamer.

* To whom correspondence should be addressed.

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Detailed study of the structure of DNA ligands is indispensable for effective application of aptamers as inhibitors of *Taq* DNA polymerase. After preliminary experiments aptamer TQ21 has been chosen for this investigation.

The objectives of this study were as follows: to define the secondary structure of DNA ligand to *Taq* DNA polymerase; to reveal functionally important elements of the aptamer that are involved in the specific interaction with the enzyme. The aim of the study was the design of an effective inhibitor of *Taq* DNA polymerase. DNA ligand can be used both for the structural studies of the enzyme and for improvement of the specificity of PCR.

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized (JSC Syntol, Russia) on an ASM 102U automatic DNA synthesizer (Novosibirsk, Russia). *Taq* DNA polymerase (M_r = 94 kD) was from Amersham (USA), T4 polynucleotide kinase was from Promega (USA), [γ-^{32}P]ATP was from Isotope (Obninsk, Russia) and was used for labeling of DNA fragments.

Measurement of IT_{50}. A model system proposed by Dang and Jayasena [19] was used for measurement of IT_{50}. One picomole of *Taq* DNA polymerase (1 unit of activity) was mixed with 1 pmol of aptamer in 10 μl of reaction buffer (20 mM Tris-acetate, pH 8.3, 10 mM $(NH_4)_2SO_4$, 75 mM KOAc, 2.5 mM $MgCl_2$) in the presence of dNTP mixture (0.2 mM each). Then 1 pmol of radiolabeled at 5′-end by [γ-^{32}P]ATP model oligonucleotide (5′-ATG-CCT-AAG-TTT-CGA-ACG-CGG-CTA-GCC-AGC-TTT-TGC-TGG-CTA-GCC-CGC-T-3′) was added and the mixture was incubated for 30 min at one of four temperatures (30, 35, 40, 45°C). The reaction was terminated by addition of 1 μl of EDTA solution (0.5 mM/ml), pH 8.0. The mixture was analyzed by electrophoresis in 10% denaturing polyacrylamide gel (acrylamide–bis-acrylamide 19 : 1 (v/w), 50 mM Tris-borate EDTA, and 7 M urea). The positions of ^{32}P-labeled compounds were determined by radioautography. The extent of reaction course (α) was assessed using the formula $\alpha = I_p/(I_p + I_o)$, where I_p is the intensity of the radioactive counts of the reaction product, I_o is the intensity of the radioactive counts of the model oligonucleotide that was not consumed in the reaction. The results were used to estimate the temperature interval within which the extend of reaction course was close to 50%. Twelve points were chosen within this interval. The value of α was measured for each point. The temperature where α was equal to 50% (IT_{50}) was assessed by a graphic method as a result of an approximating function of experimental data at 12 temperatures.

Covalent shift of *Taq* DNA polymerase to DNA aptamer containing 5-bromo-2′-desoxyuridine residue. *Taq* DNA polymerase (500 pmol) was added to 1 pmol of DNA (100,000 cpm) in reaction buffer containing 20 mM Tris-HCl, pH 7.0, 70 mM KCl, and 20 mM $MgCl_2$ (the final volume of reaction mixture was 10 μl). The reaction mixture was incubated for 20 min at room temperature (20°C) and then for 10 min at 8°C. DNA–protein complexes were exposed afterwards to UV light at 302 nm for 30 min. Then 5 μl of the solution containing 0.05 M Tris-HCl, pH 6.8, 2% SDS, 2% β-mercaptoethanol, 30% glycerol, and marker dyes xylene cyanole (0.1%) and bromophenol blue (0.1%) were added to the samples. The samples were heated for 5 min at 90°C and analyzed in 12.5% SDS-PAGE [21].

RESULTS

Study of the secondary structure of oligonucleotides—aptamers for *Taq* DNA polymerase. The study of the secondary structures of 32 DNA aptamers of two families [19] was started by the estimation of the models of secondary structures characterized by the minimal internal energy. The calculation was carried by out by the algorithm of Zuker and Stingler [22]. It was shown that all studied aptamers were able to form one, two, or three loops. Secondary structure with several loops was typical for the members of the TQ30 family (14 aptamers out of 16). In the TQ21 family 8 out of 16 aptamers formed a hairpin structure with one homologous loop.

Oligonucleotide TQ21, which inhibited *Taq* DNA polymerase most effectively, is among the 8 above-mentioned aptamers. We distinguished three principal fragments in the secondary structure of this aptamer: single-stranded 5′-end, double-stranded stem consisting of two fragments that are separated by an internal loop, and loop fragment (Fig. 1).

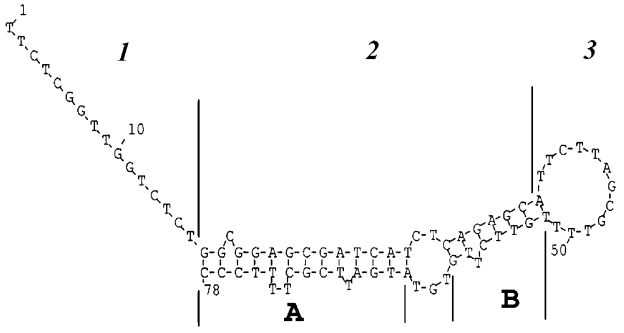

Fig. 1. Model of the secondary structure of TQ21 aptamer. Structural elements: *1*) single-stranded 5′-end; *2*) double-stranded region consisting of two fragments (A and B) separated by an internal loop; *3*) fragment of the loop.

The existence in solution of the calculated hairpin structure for the oligonucleotide TQ21 was proven by the methods of temperature denaturation [23] and chemical modification followed by cleavage of single-stranded DNA [24].

It is well known that in solutions at low concentrations (0.1-2.5 mM) oligonucleotide hairpin structures have higher melting temperatures than oligonucleotide duplexes that correspond to their double-stranded fragments. This effect deals with the stabilizing influence of the loop. In addition, the melting temperature of a hairpin is independent of the concentration of DNA ligand.

Thus, comparison of thermal melting for TQ21 and a mixture of oligonucleotides that form the double-stranded fragment of TQ21 (TQ21-duplex) (Table 1) was used to assess the interaction between heterocyclic bases in the aptamer, both within one molecule and between molecules (oligonucleotide sequences in TQ21 and TQ21-duplex are presented in Table 2).

TQ21-duplex was formed by slow renaturation (from 90 to 30°C) of equimolar quantities of each chain of the double-stranded fragment in PCR buffer. Analysis of the temperature dependence of UV-absorption for TQ21 and TQ21-duplex demonstrated that the melting curves of both structures were ascending. TQ21 was characterized by higher cooperativity than TQ21-duplex. This observation provided evidence to suggest that intramolecular interactions between bases prevailed in TQ21 aptamer.

Table 1. Melting temperatures (T_m) and energies of duplex formation at 37°C (ΔG°_{37}) for DNA ligands and DNA duplexes

Oligonucleotide system	Concentration of each strand, μM	T_m, °C	ΔG°_{37}, kcal/mol
TQ21	0.1	41 ± 2%	
	0.5	41 ± 2%	−0.6 ± 2%
	1.0	41 ± 4%	
	2.5	41 ± 0.5%	
TQ21	0.1	19 ± 2%	
duplex	0.5	20 ± 2%	6.2 ± 2%
	1.0	21 ± 4%	
	2.5	23 ± 0.5%	
TQ21-11	0.1	87 ± 2%	
	0.5	87 ± 2%	−18.6 ± 2%
	1.0	87 ± 4%	
	2.5	87 ± 0.5%	
TQ21-11	0.1	70 ± 2%	
duplex	0.5	72 ± 2%	−16.4 ± 0.5%
	1.0	73 ± 4%	
	2.5	76 ± 0.5%	

Note: ΔG°_{37} and T_m are presented as approximate values obtained from several independent curves of temperature denaturation of samples. Data were obtained in the following buffer: 20 mM Tris-HCl, 70 mM KCl, 2 mM $MgCl_2$, pH 7.0. Each experiment was repeated three times.

The results of the measurement of the melting temperature (T_m) and the energy of duplex formation at 37°C (ΔG°_{37}) for TQ21 and TQ21-duplex are presented in Table 1. It was shown that the melting temperature for TQ21 was 41°C and was independent of the concentration of DNA ligand. The melting temperature for TQ21-duplex was significantly lower, 23°C at the maximal of examined concentrations of the duplex (2.5 μM). Hence TQ21 forms between molecules in solution an intramolecular structure that is more stable than TQ21-duplex.

The method of chemical modification provides alternative evidence of the existence in solution of the calculated model of the secondary structure. It is known that potassium permanganate and hydroxylamine attack a pyrimidine perpendicularly to the plane of the heterocyclic base. Thus, only heterocyclic bases that are not involved in the formation of double helix can be modified. Analysis of the degradation products of TQ21 oligonucleotide revealed that cytosines in positions 19, 42, and 47 and thymidines in positions 40, 41, 43, 44, and 49-51 from the 5′-end reacted with chemical reagents most actively. Base T52 reacted slightly weaker. Base C19 corresponds to the looping of fragment *2* of the secondary structure of TQ21 (Fig. 1). All other nucleotides that were subjected to modification correspond to the loop of this oligonucleotide. Thus, we proved by chemical modification the existence in solution of the calculated hairpin structure of the oligonucleotide TQ21.

Search for structural elements of DNA ligand that influence the efficiency of binding to *Taq* DNA polymerase. The next step of this investigation consisted of elucidation of a fragment in TQ21 structure bound to *Taq* DNA polymerase and of determination of the structural elements that influence the strength of the aptamer complex with the enzyme.

It is known that the inhibitory effect of TQ21 aptamer on DNA polymerase activity of the enzyme depends on the temperature [20]. Therefore, we applied the following parameter for the assessment of the dependence of the inhibiting effect of DNA ligand on the temperature: the temperature of 50% reaction course of polymerization of the model oligonucleotide (IT_{50}). The model system of Dang and Jayasena [19] was used for measurement of IT_{50} (see "Materials and Methods").

The results of the experiment are presented in Fig. 2. An example of complete inhibition of the enzyme by the aptamer is demonstrated on lanes *1* and *5* of the radioau-

Table 2. Influence of the primary structure of DNA ligand on the temperature dependence of the inhibition of *Taq* DNA polymerase by the aptamer

Oligo-nucleotide	Sequence (5′-3′)[a]			IT_{50}, °C[b]
TQ21	TTCTCGGTTGGTCTCTGGCGGAGCGATCATCTCAGAGCA	TTCTTAGCGTTT	TGTTCTTGTGTATGATTCGCTTTTCCC	37.0
TQ21-1	GGCGGAGCGATCATCTCAGAGCA	TTCTTAGCGTTT	TGTTCTTGTGTATGATTCGCTTTTCCC	37.3
TQ21-2	GCGATCATCTCAGAGCA	TTCTTAGCGTTT	TGTTCTTGTGTATGATTCGC	38.2
TQ21-3	GCCGCTCAGAGCA	TTCTTAGCGTTT	TGTTCTTGTGTCGGC	30.7
TQ21-4	CGGTCGGCTCGGGGCA	TTCTTAGCGTTT	TGCCCCGAGCCGACCG	35.1
TQ21-5	GGTCGGCTCGGGGCA	TTCTTAGCGTTT	TGCCCCGAGCCGACC	25.3
TQ21-6	TCGGCTCGGGGCA	TTCTTAGCGTTT	TGCCCCGAGCCGA	25.1
TQ21-7	CAGAGCA	TTCTTAGCGTTT	TGTTCTG	—
TQ21-8	GGGCG	TTCTTAGCGTTT	CGCCC	—
TQ21-9	GCGATCATCTCAGAGCA	TTCTTAGCGTTT	TGTTCTGAGATGATCGC	42.1
TQ21-10	ATTAATTTAAATTAGTA	TTCTTAGCGTTT	TATTAATTTAAATTAAT	41.7
TQ21-11	GCGGTCGGCTCGGGGCA	TTCTTAGCGTTT	TGCCCCGAGCCGACCGC	45.0
TQ21-11 non	GCGGTCGGCTCGGGGCA	TTTTTTTTTTTT	TGCCCCGAGCCGACCGC	31.5
TQ21 duplex				
first strand	GGCGGAGCGATCATCTCAGAGCA			—
second strand	CCCTTTTCGCTTAGTATGTGTTCTTGT			—
TQ21-11 duplex				
first strand	GCGGTCGGCTCCGGGGCA			—
second strand	TGCCCCGAGCCGACCGC			—

[a] The sequence of the loop is printed within a frame.
[b] Error of IT_{50} measurement didn't exceed 1°C.

tograph. The bands of both initial oligonucleotide and reaction product are present on lanes *2* and *9*. This occurs when the enzyme activity is partly inhibited. On lane *1* the only signal comes from 64-nucleotide-long product of polymerization reaction; hence, in this case DNA ligand did not interact with the enzyme.

To estimate the structural elements of DNA ligand that influence the binding to the enzyme, we synthesized the variety of oligonucleotides presented in Table 2. The oligonucleotides differed from the initial TQ21 in the absence of the single-stranded 5′-end (TQ21-1), length and nucleotide composition of the double-stranded fragment including the absence of looping (TQ21-2 to TQ21-8), and the absence of the loop (TQ21-11 duplex). Each of these oligonucleotides was tested for ability to inhibit *Taq* DNA polymerase.

According to the experimental data (Fig. 2, Table 2), the oligonucleotide without single-stranded 5′-end (TQ21-1) interacted with *Taq* DNA polymerase and bound the enzyme at 35°C even more effectively than the initial TQ21 (Fig. 2). For TQ21-1 IT_{50} was equal to 37.3, that is 0.3°C higher than for TQ21. Therefore, the single-stranded 5′-end is not essential for specific interaction of DNA ligand with the enzyme. The aptamer without the single-stranded 5′-end is 62-nucleotides long. This result comes in agreement with the data indicating that the 51-nucleotide-long fragment of DNA ligand TQ21 conserved ability to inhibit *Taq* DNA polymerase [20].

We then assessed the crucial length of the double-stranded fragment of DNA ligand (DNA ligands TQ21-1

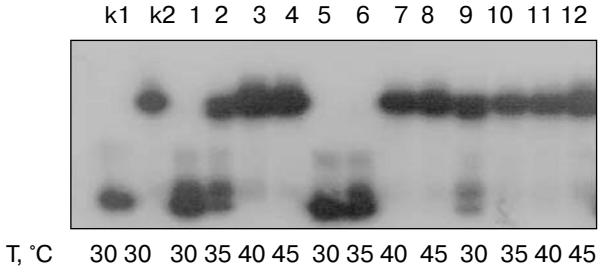

Fig. 2. Influence of temperature on binding of DNA ligands to *Taq* DNA polymerase. Lanes: k1) initial 49-nucleotide-long oligonucleotide; k2) 64-nucleotide-long oligonucleotide, product of polymerization reaction in the absence of DNA ligand; *1-4*) TQ21; *5-8*) TQ21-1; *9-12*) TQ21-11non.

to TQ21-8 in Table 2). Oligonucleotides TQ21-1 and TQ21-2 had similar IT_{50} values. However, the reduction of the double-stranded region by only one nucleotide (oligonucleotide TQ21-4 in comparison to TQ21-2) led to a decrease in IT_{50} by 3.1°C. Thus, the crucial size of the double-stranded fragment of DNA ligand is 17 base pairs.

It is interesting that TQ21-9 aptamer, which differed from TQ21-2 in the absence of looping, bound the enzyme with the same efficiency. This means that the internal loop of region *2* is not essential in the structure of the optimized DNA ligand.

We demonstrated that the nucleotide sequence of the double-stranded region had no influence on the strength of the DNA–protein complex by using oligonucleotides with different composition of this region (TQ21-9 to TQ21-11). However, the maximal IT_{50} value was measured for oligonucleotide with GC-rich double-stranded region (TQ21-11) (Table 2).

Afterwards, we assessed if the presence of the loop (region *3* in Fig. 1) in the aptamer was essential for inhibitory capacities of DNA ligand. In these experiments a mixture of oligonucleotides that corresponded to the double-stranded fragment of DNA ligand TQ21-11 (TQ21-11 duplex, Table 2), was studied in the model system of Dang and Jayasena [19].

The IT_{50} value for TQ21-11 duplex was 30.7°C, which is much lower than IT_{50} = 45 for DNA ligand TQ21-11 with a loop. Since there was no significant difference between melting temperatures of these oligonucleotides (T_m = 70°C for TQ21-11 duplex and T_m = 87°C for TQ21-11), the reduction of IT_{50} value could not be due to the lower thermostability of TQ21-11 duplex. Hence, the presence of the loop is necessary for inhibition of the enzyme by DNA ligand.

Thus, we established that for TQ21 DNA ligand the region of interaction with *Taq* DNA polymerase consisted of fragments *2* and *3* of the secondary structure (Fig. 1).

Study of the influence of the primary structure of the loop on the specificity of DNA–protein interaction. As mentioned earlier, the conservative region for the representatives of TQ21 family correspond to the fragment of the loop. The sequence of this fragment can be represented as follows:

–(A/T)T-PyPyN-NPuC-GTT-TTG–,

where N is any nucleotide, Py is pyrimidine, Pu is purine, (A/T) is A or T.

It is possible that just this fragment in the aptamer structure is crucial for the inhibiting properties of DNA ligands. To check this suggestion we synthesized an oligonucleotide with all nucleotides of the loop changed for thymidines (DNA ligand TQ21-11non in Table 2). The study of binding of such oligonucleotide to *Taq* DNA polymerase demonstrated that IT_{50} value for TQ21-11non was 31.5, which was much lower than IT_{50} = 45 for oligonucleotide with the initial sequence of the loop TQ21-11, but was close to IT_{50} = 30.7 for TQ21 duplex. A large decrease in the strength of interaction of DNA ligand TQ21-11non with the enzyme indicates that the nucleotide sequence of the loop determines the specificity of this interaction.

A number of oligonucleotides (mut 18-29) with replacement of one of the nucleotides in the loop (T instead of A, C instead of G) were synthesized to establish what heterocyclic bases were involved in the formation of spatial structure responsible for the interaction with the enzyme.

These oligonucleotides are presented in Table 3. It demonstrates that any replacement led to reduction of the inhibitory properties of the DNA ligand. The minimal decrease in inhibition was observed when the bases in positions 23 and 29 were replaced. Since the inhibitory properties even of the oligonucleotide mut 23 (C) were not significantly changed, we suggest that the base 23 of the loop could be replaced by any nucleotide. This suggestion was confirmed by the analysis of the sequences of the members of the TQ21 family.

Since base 29 that closes the loop fragment did not significantly influence the inhibition, it was interesting to estimate what would happen with the activity of the aptamer after elimination of this nucleotide from its

Table 3. Influence of the nucleotide sequence in the loop of DNA ligand on the temperature dependence of *Taq* DNA polymerase inhibition by the aptamer

Oligonucleotide	Loop sequence	IT_{50}, °C
TQ21-11	..TTC-TTA-GCG-TTT-..	45
Mut 18[a]	..**a**TC-TTA-GCG-TTT-...[b]	30.4
Mut 19	..T**a**C-TTA-GCG-TTT-...	30.7
Mut 20	..TT**g**-TTA-GCG-TTT-...	30.1
Mut 20 (T)	..TT**t**-TTA-GCG-TTT-...	27
Mut 21	..TTC-**a**TA-GCG-TTT-...	32
Mut 22	..TTC-T**a**A-GCG-TTT-...	32.4
Mut 23	..TTC-TT**t**-GCG-TTT-...	38.2
Mut 23 (C)	..TTC-TT**c**-GCG-TTT-...	35
Mut 24	..TTC-TTA-**c**CG-TTT-...	28.1
Mut 25	..TTC-TTA-G**g**G-TTT-...	31.9
Mut 26	..TTC-TTA-GC**c**-TTT	26.5
Mut 27	..TTC-TTA-GCG-**a**TT	27.1
Mut 28	..TTC-TTA-GCG-T**a**T	26.4
Mut 29	..TTC-TTA-GCG-TT**a**	38.6

[a] Figure in the abbreviation of the oligonucleotide corresponds to the position number starting from the 5′-end of the oligonucleotide.

[b] The changed nucleotide is indicated with bold type.

sequence (TQ21-11-minus) or after introduction of an additional upstream nucleotide (TQ21-11-plus).

The study of the binding of oligonucleotides with changed loop length (TQ21-11-minus and TQ21-11-plus) to the enzyme showed that the inhibitory properties of these DNA ligands were significantly decreased (IT_{50} values were 25.6 and 25.7°C, respectively). Therefore the optimal length of the loop in the aptamer was 12 nucleotides.

According to the results on the structural peculiarities of DNA ligands to *Taq* DNA polymerase, TQ21-11 was chosen as the most effective inhibitor of the enzyme. The affinity of this oligonucleotide to the enzyme was 5-fold higher than that of the initial aptamer TQ21. The values of dissociation constants were measured for these two oligonucleotides by the standard method of binding to nitrocellulose filters. They were equal to 7 ± 1 pM for TQ21-11 and 36 ± 3 pM for TQ21.

The secondary structure of the optimized DNA ligand TQ21-11 was tested by evaluation of the thermal stability of the oligonucleotide and of TQ21-11 duplex (Table 1) and also by chemical modification followed by cleavage of the single-stranded DNA fragments. The results confirmed the hairpin structure.

Detection of thymine residues potentially involved in the specific interaction with *Taq* DNA polymerase. To establish what thymines in the structure of DNA ligands were close to *Taq* DNA polymerase, we used the method of photo-inducible binding. A number of oligonucleotides presented in Table 4 were synthesized. In the TQ21-11 sequence one of the thymidines in the loop (DNA ligands No. 1-4, 6-9 in Table 4) or in the double-stranded region (Nos. 9 and 11 in Table 4), and also adenosine 23 (No. 5) were replaced by 5-bromo-2′-deoxyuridine. DNA fragment without modified nucleotides was used for the control of the specificity of covalent binding.

Table 4 shows that the maximal percent of shift (7.4%) was observed for oligonucleotide with the replacement in position 23 from the 5′-end. This result provided evidence for the suggestion that this base is in direct contact with the enzyme. Interestingly, such replacement did not modify the ability of the aptamer to inhibit *Taq* DNA polymerase (IT_{50} = 50 was even slightly higher than in the control).

Oligonucleotide with dodecathymidine loop and 5-bromo-2′-deoxyuridine in the same position 23 (No. 10 in Table 4) also caused a high percent of covalent shift (5.9%), but inhibited the enzyme much worse than other DNA ligands from this group. It is interesting that in oligonucleotide No. 10 the loop fragment between nucleotides 18 and 22 remained almost unchanged in comparison to TQ21-11. Possibly, the structure of this fragment determined the approach between the loop of the aptamer and the protein. However, the replacement of bases 24-29 in the loop led to large reduction in inhibition.

The data of Table 4 indicate that introduction of the modified base instead of T10, T27, T29, and T30 (Nos. 6, 8, 9, and 11 in Table 4) led to the reduction of the enzyme

Table 4. Photo-inducible covalent shift of DNA ligands containing 5-bromo-2′-deoxyuridine to *Taq* DNA polymerase

No.	Fragment of the sequence of DNA ligands	% of covalent shift[a]	IT_{50},°C
1	5'- ..... A***br⁵dU***- *T-C- T- T - A -GCG- T- T- T-* T..... -3'[b]	1.2 ± 0.4	42
2	5'- ..... A- *T-* ***br⁵dU****C- T- T- A- GCG- T- T- T-* T..... -3'	1.4 ± 0.7	45
3	5'- ..... A- *T- T-C* ***br⁵dU****- T- A -GCG- T- T- T-* T..... -3'	1.6 ± 0.5	45
4	5'- ..... A- *T- T-C - T-* ***br⁵dU****-A-GCG- T- T- T-* T..... -3'	2.6 ± 0.8	45
5	5'- ..... A- *T- T- C- T- T-* ***br⁵dU****GCG- T- T- T-* T..... -3'	7.4 ± 1.2	50
6	5'- ..... A- *T- T-C- T- T- A- GCG* ***br⁵dU****- T- T-* T..... -3'	1.9 ± 0.6	40
7	5'- ..... A- *T- T-C- T- T- A- GCG- T* ***br⁵dU****- T-* T..... -3'	1.7 ± 0.5	45
8	5'- ..... A- *T- T-C- T- T- A- GCG- T- T-* ***br⁵dU****-* T..... -3'	1.4 ± 0.3	40
9	5'- ..... A- *T- T-C- T- T- A- GCG- T- T- T-* **br⁵dU**...-3'	1.9 ± 0.4	36
10	5'- ..... A- *T- T-* ***Tbr⁵dU****- T- T- T TT- T- T- T-* T..... -3'	1.7 ± 0.3	31.5
11	5'- ...**br⁵dU**..A- *T- T- C- T- T- A- GCG- T- T- T-* T..... -3'[c]	5.9 ± 0.8	38.2
C[d]	5'- ..... A- *T- T- C- T- T- T- A- GCG- T- T- T-* T .... -3'	0	45

[a] Each experiment was repeated three times to calculate % of covalent shift.
[b] Sequences of loops (italics) and first nucleotides of the double-stranded fragments of DNA ligands.
[c] Thymidine in position 10 from the 5′-end is changed to Br^5dU.
[d] Control is TQ21-11.

inhibition by the oligonucleotide. This result of thymidine replacement by 5-bromo-2′-deoxyuridine could be explained by possible involvement of the methyl group of thymidine in the formation of hydrophobic links with amino acid residues of the enzyme.

The results can be used to estimate what nucleotides in the DNA ligand are involved in the direct contact with the enzyme. However, this information is not sufficient for the explanation of the mechanism of inhibition of *Taq* DNA polymerase by the aptamer.

DISCUSSION

The results of our studies of the structure of DNA ligand provided the possibility to reveal the most significant structural elements of the aptamer and to optimize the desired properties.

Formation of the hairpin secondary structure is necessary for expression of the inhibitory properties by the oligonucleotide TQ21-11.

Data in the literature indicate that the presence of an intramolecular hairpin is typical for many DNA ligands [25-28]. The aptamers to thrombin that block blood clotting were studied most intensively [29-31], as well as the aptamers to L-selectin, which inhibit migration of human lymphocytes in mice [32, 33].

The design of DNA ligands with high affinity to various target molecules is of scientific and practical importance. The mechanism of protein binding to specific sequences of double-stranded DNA (these proteins are mainly transcription factors) is the better-studied model [34-37]. However, the secondary structures of the single-stranded DNA fragments in complexes with proteins are somewhat investigated.

There are several methodological approaches for the study of DNA–protein complexes. The photo-inducible chemical shift of DNA ligand to proteins is one of these. This method can be used to analyze what amino acid residues are involved in the formation of DNA–protein complex. The data in the literature led to the suggestion that 5-bromo-2′-deoxyuridine incorporated in the aptamer structure was able to form covalent links with the residues of aromatic amino acids (Phe, Trp, Tyr) and His [32, 38].

Work on the optimization of DNA aptamers to *Taq* DNA polymerase has not only scientific value. The results have been applied in the practice of PCR. The addition of DNA ligand to the reaction mixture prevented the synthesis of nonspecific products because it ensured the hot-start of PCR after the reaction mixture had reached the temperature of dissociation of the enzyme–aptamer complex.

As indicated earlier, the affinity of the optimized ligand TQ21-11 to the enzyme was 5-fold greater than that of the initial aptamer (K_d values were 7 ± 1 pM for TQ21-11, 36 ± 3 pM for TQ21, and 40 ± 2 pM for TQ30). In addition, TQ21-11 inhibited *Taq* DNA polymerase over a broader temperature range (25-45°C) than aptamers proposed by NeXstar Pharmaceuticals (USA) [19, 20]. Therefore TQ21-11 is more effective for the improvement of PCR specificity and sensitivity. We demonstrated that DNA ligand TQ21-11 may also be successfully used for RT-PCR in a single tube (*Biotekhnologiya* (2002) **5**, in press).

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