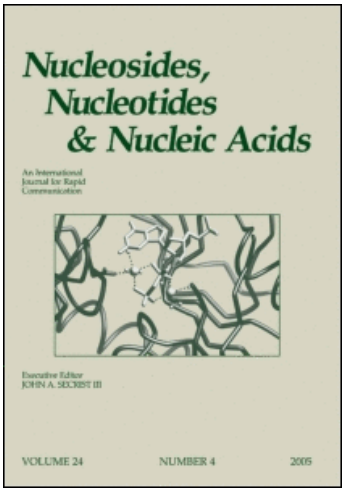

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Invasion of Strongly Binding Oligonucleotides into tRNA Structure

V. Petyuk[ab]; R. Serikov[c]; V. Tolstikov[c]; V. Potapov[c]; R. Giege[d]; M. Zenkova[ab]; V. Vlassov[ab]
[a] Laboratory of Nucleic Acids Biochemistry, Novosibirsk Institute of Bioorganic Chemistry, Novosibirsk, Russia [b] Novosibirsk State University, Russia [c] Laboratory of Structure and Function of Human Genes, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia [d] Département "Mécanismes et Macromolécules de la Synthèse Protéquie et Cristallogènese", UPR 9002 'SMBMR', Institut de Biologie Moléculaire et Cellulaire du CNRS, Strasbourg Cédex, France

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INVASION OF STRONGLY BINDING OLIGONUCLEOTIDES INTO tRNA STRUCTURE

V. Petyuk[1),2)], R. Serikov[2)], V. Tolstikov[3)], V. Potapov[3)], R. Giegé[4)], M. Zenkova[*,1),2)], V. Vlassov[1),2)]

[1)]Laboratory of Nucleic Acids Biochemistry, Novosibirsk Institute of Bioorganic Chemistry, 8 Lavrentiev avenue, 630090, Novosibirsk, Russia
[2)]Novosibirsk State University, 2, University av., 630090, Russia
[3)]Laboratory of Structure and Function of Human Genes, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Ul. Miklukho-Maklaya 16/10, Moscow 117871, Russia
[4)] Département "Mécanismes et Macromolécules de la Synthèse Protéique et Cristallogenèse", UPR 9002 'SMBMR', Institut de Biologie Moléculaire et Cellulaire du CNRS, 15 rue René Descartes, 67084 Strasbourg Cédex, France

ABSTRACT: Interaction of yeast $tRNA^{Phe}$ with oligodeoxyribonucleotides containing 5-methylcytosine, 2-aminoadenine, and 5-propynyl-2'-deoxyuridine was investigated. The modified oligonucleotides show increased binding capacity although the association rates are similar for the modified and natural oligonucleotides. The most pronounced increase in association constant (70 times) due to the incorporation of the strongly binding units was achieved in the case of oligonucleotide complementary to the sequence 65-76 of the $tRNA^{Phe}$.

INTRODUCTION

The use of oligonucleotides (ONs) as diagnostic probes and antisense agents is based upon the Watson-Crick base-pairing of the oligomers to complementary nucleic acid sequences. Identification of optimal target nucleotide sequences for oligonucleotides in RNAs represents a

problem, because under physiological conditions RNAs are folded in compact structures in which only minor fractions of sequence are open single-stranded regions available for hybridization with ONs [1, 2]. However, experimental studies indicate, that in some cases, oligonucleotides can bind to folded RNAs by invading into certain elements of their structure [3-7]. Thus, it was found that complementary oligonucleotides can invade the ends of homologous duplexes [3] or can bind to some sequences in tRNAs as in yeast $tRNA^{Phe}$ in spite of the tight folding of this molecule [4, 5]. Particularly efficient binding was observed with the 3'-part of this RNA [5-7]. We have found, that oligonucleotides can invade into the tRNA structure under physiological conditions, if the complementary sequence is the 3'-terminal part of the tRNA including the single-stranded $ACCA_{OH}$-sequence at the 3'-end, that is important for initial binding and initiation of the strand displacement process [6, 7].

A number of modifications have been developed for improving affinity of oligonucleotides to their complementary sequences [8-15]. We have investigated the hybridization and strand invasion properties of modified oligonucleotides designed to have increased hybridization capacity. These strongly binding oligonucleotides (SB-ONs) containing 2-aminoadenine, 5-methylcytosine, and 5-propynyl-uracile instead of natural heterocyclic bases, form more stable hybrids with complementary RNA sequences. Each substitution with 2-aminoadenine and 5-methylcytosine increases the melting temperature (T_m) of the duplex by approximately 1°C [16]. In the case of C5-(3-methoxypropynyl) substituted 2'-deoxyuridine, the T_m increase was 1.7°C per modified dU residue [13, 17]. We have found that hybridization of modified oligonucleotides with target sequence in yeast $tRNA^{Phe}$ is thermodynamically favored due to increased binding capacity, but association occurs with similar rate as compared to natural ONs. The most pronounced increase in association constant (70 times) as compared to the natural ON was observed with oligonucleotide SB-ON-C containing 7 modified bases (4 T*, 2 A*, and 1 C*, see Fig. 1) complementary to the sequence 65-76 of $tRNA^{Phe}$. We have found, that the shortest modified oligonucleotide capable of invading the $tRNA^{Phe}$ acceptor stem is a 12-mer, complementary to the tRNA sequence 65-76.

EXPERIMENTAL

Materials

Yeast $tRNA^{Phe}$ was a generous gift of G. Keith from Institut de Biologie Moléculaire et Cellulaire du CNRS, Strasbourg, France. T4 RNA ligase was purchased from Boehringer

Mannheim, 5'-[^{32}P]-pCp (4000 Ci/mmol) was from Biosan Co. (Russia). Yeast tRNA^{Phe} was labeled at the 3'-terminus with 5'-[^{32}P]-pCp and T4 RNA ligase as described in [6].

Oligonucleotides

Oligonucleotides TGGTGCGAATTCT (ON-B), TGGTGCGAATTC (ON-C), GTGCGAATTCTGT (ON-F), and TGCGAATTCTGT (ON-G) were synthesized by standard phosphoramidite chemistry and purified by ion-exchange and reverse-phase HPLC. The oligonucleotides were homogenous as assayed by 20% PAGE in denaturing conditions followed by staining with Stains-All [18].

Suitably protected 5-methyl-2'-deoxycytidine and 2-amino-2'-deoxyadenosine 3-cyanoethyldiisopropyl phosphoroamidites were prepared as described earlier [16, 19]. 5-Propynyl-2'-deoxyuridine 3-cyanoethyldiisopropyl phosphoroamidite was purchased from Glen Research Corporation, USA. Oligonucleotides (SB-ON for oligonucleotides with modified nucleobases) SB-ON-B, SB-ON-C, SB-ON-F, and SB-ON-G (sequences of the oligonucleotides are the same as for ON-B, ON-C, ON-F, ON-G, respectively) were synthesized on the controlled pores glass support on a ASM-102 U DNA synthesizer (Biosan Ltd.). After the cleavage from support and deprotection by aqueous ammonia at 50°C for 5 hours, the oligonucleotides were purified by reversed phase HPLC on a Sepharon C-18 column followed by removal of 4,4-dimethoxytrityl group and desalting. Purity of oligonucleotides was confirmed by analytical reversed phase HPLC and by electrophoresis on 18% denaturing polyacrylamide gel, followed by oligonucleotides visualization using Stains-All.

Hybridization of tRNA^{Phe} with oligonucleotides

Hybridization of oligonucleotides to tRNA^{Phe} was monitored by gel mobility shift assay [6, 7]. Prior to hybridization, 3'-[^{32}P]-tRNA (final concentration in the mixture, $5 \cdot 10^{-7}$ M) was heated at 90°C in 3 μl of water for 1 min, then cooled down and incubated at 20°C for 10 min. The 5x hybridization buffer (1x: 50 mM HEPES-KOH, pH 7.5, 200 mM KCl, 0.1 mM EDTA) was added and the solution was again incubated for 20 min at 20°C. ON and SB-ON solutions ranging in concentration from $1 \cdot 10^{-6}$ M to $1 \cdot 10^{-3}$ M were added and the final mixtures (volume of each reaction mixture was 10 μl) were incubated at 20°C. After incubation, 8 μl of loading buffer (50% glycerol, 0.025% bromophenol blue, 0.025% xylene cyanol) was added to each 10 μl probe and the probes were electrophoresed at 4°C in native

10% PAGE using 100 mM Tris-borate running buffer, pH 8.3, containing 1 mM EDTA, for 6 h at 10 V/cm. To get quantitative data, the gels were dried, radioactive bands were cut out of the gel, and their radioactivity was determined by Cherenkov's counting.

Phosphodiesterase hydrolysis of SB-ON-B

Snake venom phosphodiesterase hydrolysis was used for production of a random mixture of successively 3'-end shortened strong binding oligonucleotide (SB-ON-B). Briefly, snake venom phosphodiesterase was added to 100 µl of 50 mM Tris-HCl, pH 8.3, containing 5'-[^{32}P]-end labeled SB-ON-B diluted with cold SB-ON-B up to a concentration of 530 µM. The digestion was carried out at 37°C for 30 min. Hydrolysis was stopped by cooling the reaction mixture in ice followed by two successive extractions with equal volumes of phenol saturated with water and extraction with chloroform. The random mixture of oligonucleotides was precipitated by addition of 1 ml of 2% lithium perchlorate in acetone, centrifuged, washed with acetone, dissolved in 10 µl MilliQ water, and analyzed by electrophoresis in 20% polyacrylamide gel under denaturing conditions.

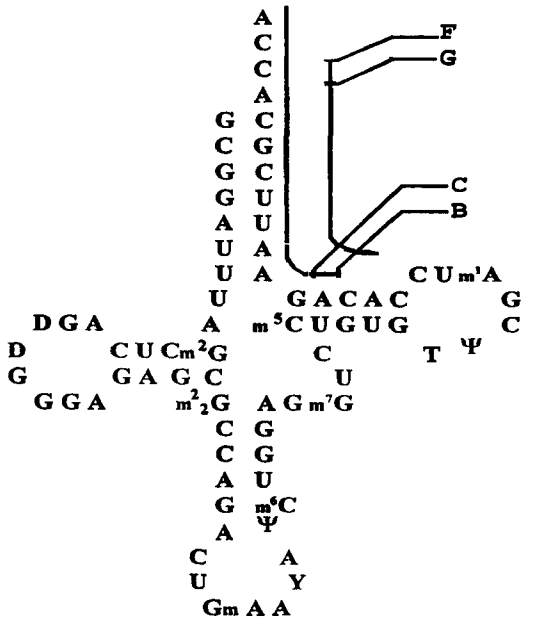
Identification of the SB-ONs capable of binding with yeast tRNA^{Phe}

The experimental procedure is shown in Fig. 4A. For identification of SB-ONs capable of binding to tRNA^{Phe}, partial phosphodiesterase hydrolysates of the 5'-end labeled SB-ON-B in concentration 20, 80 or 240 µM were incubated with 2 µM tRNA^{Phe} at 20°C for 4 h in 50 mM HEPES-KOH, pH 7.5, 0.2 M KCl, 0.5 mM EDTA (final volume 10 µl). After incubation, 8 µl loading buffer were added to the reaction mixture. Products of the hybridization reaction and non-bound oligonucleotides were separated by electrophoresis in 10% native polyacrylamide gel with 100 mM Tris-borate, pH 8.3, running buffer containing 1 mM EDTA. Gel slices containing tRNA complexed with SB-ONs were excised, 5'-end labeled ONs were eluted with water, precipitated with 2% lithium perchlorate in acetone, redissolved in gel loading buffer (4 M urea, 0.01% xylene cyanol, 0,01% bromophenol blue), and analyzed by electrophoresis under denaturing conditions in a 20% polyacrylamide gel.

RESULTS AND DISCUSSION

Design of strongly binding oligonucleotides (SB-ONs)

Synthetic oligonucleotides with increased affinity to their complementary sequences represent improved antisense agents capable to invading into RNA structures. We investigated



ON-B	TGGTGCGAATTCT
SB-ON-B	TGGTGCGAATTCT
ON-C	TGGTGCGAATTC
SB-ON-C	TGGTGCGAATTC
ON-F	GTGCGAATTCTGT
SB-ON-F	GTGCGAATTCTGT
ON-G	TGCGAATTCTGT
SB-ON-G	TGCGAATTCTGT

FIG. 1. Cloverleaf structure of yeast $tRNA^{Phe}$ [24] and antisense oligonucleotides complementary to the 3'-terminal region of the $tRNA^{Phe}$. Modified bases (see text) in the strongly binding oligonucleotides (SB-ON) are underlined. Black lines indicate complementary sequences.

the ability of strongly binding oligonucleotides (SB-ONs) to invade the tRNA structure under physiological conditions. Yeast $tRNA^{Phe}$ was used as a target as we have shown previously that some oligonucleotides can invade in the 3'-part of this molecule [6, 7]. SB-ONs contained 5-methyl-2'-deoxycytidine, 2-amino-2'-deoxyadenosine and 5-propynyl-2'-deoxyuridine instead of natural nucleosides in all positions, except for the 3'-terminal residue. The target sites for the oligonucleotides in $tRNA^{Phe}$ are shown in Fig. 1. Oligonucleotides ON-B, SB-ON-B, ON-F, and SB-ON-F were 13-mers and ON-C, SB-ON-C, ON-G, and SB-ON-G

were 12-mers, complementary to sequences 64-76 (B), 62-74 (F), 63-76 (C), and 62-73 (G) in $tRNA^{Phe}$, respectively. The target sequences comprise the terminal single-stranded $ACCA_{OH}$-sequence of the tRNA, the acceptor stem and part of the T-stem. We have shown previously [6], that complementarity to the $ACCA_{OH}$-sequence at the very 3'-end and to part of the T-stem is needed for effective binding of oligonucleotides with the 3'-part of $tRNA^{Phe}$.

We expected to achieve more efficient binding of the modified oligonucleotides (SB-ONs), because enhanced stability of duplexes formed by SB-ONs should lead to more efficient displacement of the natural complementary sequence in the acceptor stem of the tRNA. Three different strategies were applied to provide a strongly binding effect in chosen sequences [16, 17, 19, 20]. Replacement of cytosine with 5-methyl-cytosine gives an increased hydrophobicity. Replacement of adenosine with 2-amino derivative gives a possibility to form an additional third hydrogen bond in A-T base-pairs. Replacement of thymine with 5-propynyl-2'-deoxyuridine provides an enhanced stacking interaction. Oligonucleotide synthesis has been done with the use of the phosphoroamidite chemistry and did not meet any problem either with monomer preparation [19] or with oligomer synthesis / deprotection / purification procedures.

Hybridization of ONs and SB-ONs with yeast $tRNA^{Phe}$

Binding of the oligonucleotides to $tRNA^{Phe}$ was studied using gel mobility shift assay. In these experiments the excess of oligonucleotide was added to the fixed amount of tRNA: in all experiments the concentration of tRNA was $5 \cdot 10^{-7}$ M, and hybridization was performed for 4 h at 20°C to determine equilibrium association constants and for different time intervals to determine association rate constants. Fig. 2 displays the kinetics of SB-ON-B binding with tRNA and Fig. 3 represents the equilibrium binding of ONs and of SB-Ons with $tRNA^{Phe}$ at 20°C. The data evidence that most tested SB-ONs are superior to non-modified ONs with respect to hybridization properties (Fig. 3). Maximal hybridization levels of ONs were 48%, 5%, and <1% for oligonucleotides ON-B, ON-C, and ON-F, respectively. ON-G did not bind to the $tRNA^{Phe}$. With SB-ON-B, SB-ON-C, SB-ON-F, the maximal binding extents were 80%, 60%, and 5%, respectively. Binding of SB-ON-G was poor (< 1%). (Fig. 3B and Table 1). For SB-ONs the benefits in association equilibrium constants (K_x) were 1.5, 70, 1.8 times for SB-ON-B, SB-ON-C, and SB-ON-F, respectively, whereas K_x value for SB-ON-G poorly bound with tRNA was 71 M^{-1}. For SB-ON-F and SB-ON-G lacking complementarity to the CCA_{OH}-sequence, relative increase in hybridization extent (ratio binding extent SB-ON

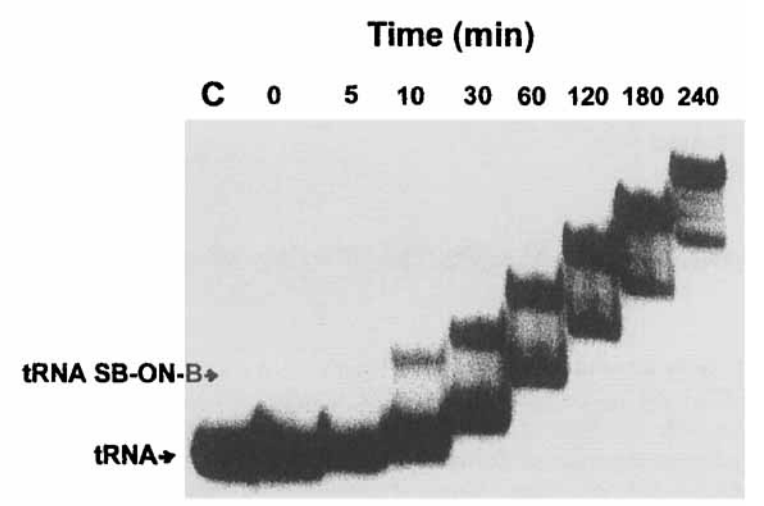


FIG. 2. Time course of SB-ON-B oligonucleotide hybridization with yeast $tRNA^{Phe}$. Autoradiograph of the 10% native PAGE after resolution of the SB-ON bound and free tRNA. The SB-ON-B and $tRNA^{Phe}$ concentrations were $7 \cdot 10^{-5}$M and $5 \cdot 10^{-7}$M, respectively. Positions of SB-ON-B bound and free tRNA are indicated by arrows. C-control tRNA incubated alone.

to binding extent of corresponding ON) was high, but overall hybridization extent remained rather poor: 5% and <1% for SB-ON-F and SB-ON-G, respectively (see Fig. 3).

The thermodynamic stabilities (ΔG) for the duplexes, formed by ONs and their linear complements, determined using the nearest-neighbors method [21, 22] were 23, 21, 21, and 20 kcal/mol for ON-B, ON-C, ON-F, and ON-G, respectively. The ΔG values for duplexes formed by ON-C and ON-F are similar and the evaluated binding constants are also close: 250 and 390 M^{-1} for ON-C and ON-F, respectively. We supposed that binding of SB-ON-C and SB-ON-F will be similar as 7 (4T*, 2A*, 1C*) and 8 (4T*, 2A*, 2C*) modified bases are incorporated in SB-ON-C and SB-ON-F, respectively, but significant increase in association constant have been observed only for SB-ON-C. This could be related to the fact, that the presence of single-stranded regions in oligonucleotide complementary sites is crucial for complex formation and is not explained by thermodynamic advantage. We suppose that the presence of a single-stranded region is a prerequisite needed for effective initiation of the hybridization process similar to nucleation of duplex formation. From the data presented, it is seen that effective initiation of the binding is important for hybridization of the strongly binding oligonucleotides as well.

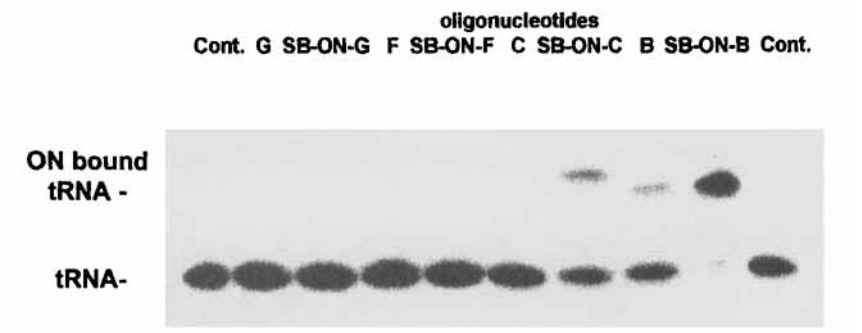


FIG. 3A. Hybridization of ONs B, C, F, G and SB- analogs with $tRNA^{Phe}$ in 50mM HEPES-KOH, pH 7.5 containing 0.2 M KCl, 0.5mM EDTA during 4 hours at 20°C. Autoradiograph of 10% native PAGE. $tRNA^{Phe}$ concentration was 0.5 μM; concentration of oligonucleotides were 70 μM. Position of ON bound and free tRNA are shown on the left. Lane «Cont.» corresponds to $tRNA^{Phe}$ incubated without oligonucleotides. G, F, C, and B designate ON-G, ON-F, ON-C, and ON-B, respectively.

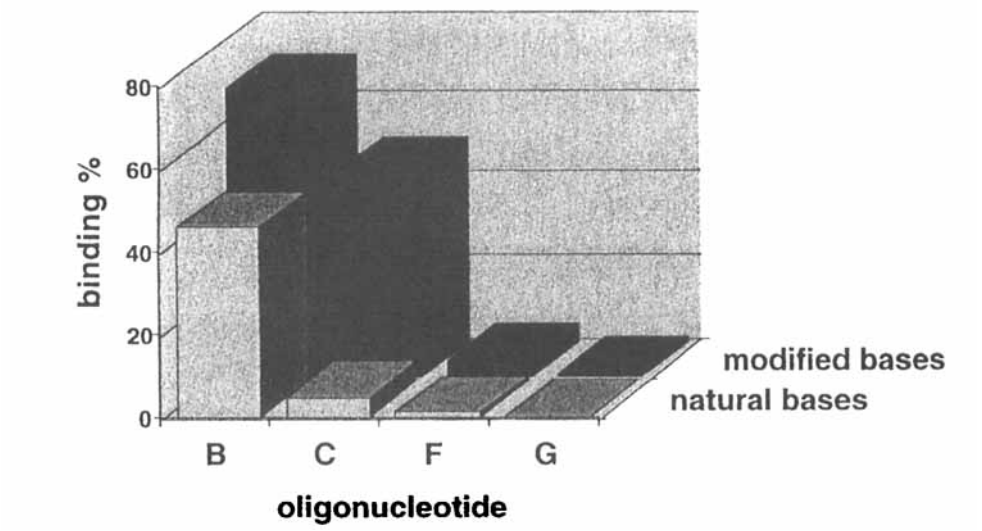


FIG. 3B. Histogram representing differences between oligonucleotide bearing canonical and modified bases in hybridization with yeast $tRNA^{Phe}$.

Binding of the ONs with the 3'-part of $tRNA^{Phe}$ is a slow process and seems to be limited by the rate of tRNA unfolding as the process is not dependent on oligonucleotide concentration [6]. The hybridization kinetics could be easily compared for ON-B and SB-ON-B. Efficiency of complex formation by other tested oligonucleotides was not high enough and

TABLE 1. Association constants for oligonucleotides ON-B, ON-C, ON-F, ON-G and SB-ON-B, SB-ON-C, SB-ON-F, SB-ON-G binding to $tRNA^{Phe}$.

Equilibrium association constants K_x (M^{-1}) *)				
Type of oligonucleotide	**B**	**C**	**F**	**G**
natural (ON)	$4.5\pm0.3\cdot10^4$	$2.5\pm0.1\cdot10^2$	$3.9\pm0.5\cdot10^2$	~0
modified (SB-ON)	$7.0\pm0.6\cdot10^4$	$1.7\pm0.3\cdot10^4$	$7.0\pm0.5\cdot10^2$	$7.1\pm0.8\cdot10^1$

Association constansts were determined using a gel-mobility shift assay as described in the section Experimental.*) Equilibrium constants were derived using a two states model

$$a(ON) = \frac{K_x * [ON]}{1 + K_x * [ON]},$$

where: *a(ON)* is the binding extent dependence on ON concentration and [*ON*] the oligonucleotide concentration.

did not allow accurate evaluation of the rate constants. We have analyzed the hybridization kinetics in a two states model assuming that both forward and reverse reactions could be affected by oligonucleotide modifications. Data are presented in Fig. 2 and Table 2. It is seen that the forward constant of hybridization (k_+) is not affected by the oligonucleotide modifications. This means that for the process of SB-ONs hybridization to tRNA, the limiting step is the tRNA unfolding, similarly as in the case of natural oligonucleotides [2, 6, 22]. In other words, the hybridization activation energy is defined by the enthalpy of tRNA structure unfolding [21, 22]. The rate constant of duplex dissociation (reverse rate constant, see Table 2) was 1.7 times higher for ON-B then for SB-ON-B, reflecting the increased stability of the heteroduplex formed by SB-ON-B with tRNA.

Identification of the minimal size of SB oligonucleotide capable of effective hybridization with $tRNA^{Phe}$

In order to identify the minimal length of SB-ONs capable of binding to the terminal sequence of $tRNA^{Phe}$, we performed the experiments schematically shown in Fig. 4A. A mixture of oligonucleotides complementary to the sequence 63-76 was produced by treatment

TABLE 2. Association rate constants for hybridization of oligonucleotides ON-B and SB-ON-B to $tRNA^{Phe}$ at 20°C.

Forward and reverse binding rate constants **)		
Type of B	**forward constant k_+ ($M^{-1}s^{-1}$)**	**reverse constant k_- (s^{-1})**
natural (ON-B)	**1.7±0.3**	**$9.4\pm0.9\cdot10^{-5}$**
modified (SB-ON-B)	**1.7±0.2**	**$5.0\pm0.5\cdot10^{-5}$**

**) forward (k_+) and reverse (k_-) constants were calculated using a two states model.

$$a(t) = \frac{(k_+ \cdot [ON])/k_-}{1 + (k_+ \cdot [ON])/k_-} \cdot (1 - e^{-t\cdot(k_+[ON]+k_-)})$$

where: $a(t)$ is the dependence of binding extent on time and $[ON]$ the oligonucleotide concentration

of 5'-[^{32}P]-SB-ON-B with snake venom phosphodiesterase. This enzyme is an exonuclease that stepwise cleaves nucleotide-5'-monophosphates from the 3'-end of nucleic acids [23]. The mixture of produced SB-ONs was incubated with $tRNA^{Phe}$ under physiological conditions at 20°C. The concentrations used (20, 80, and 240 μM) allowed to identify all SB-ONs species capable of tight binding with tRNA. Besides, under the conditions used (ONs concentrations) the hybridization rate does not depend on the SB-ON mixture concentration (not shown).

The hybridization process was monitored by separating tRNA-bound and free SB-ONs mixture by native polyacrylamide gel electrophoresis. In the conditions used, SB-ONs mixture bound to non-labeled $tRNA^{Phe}$ could be clearly distinguished from the free SB-ONs mixture and free tRNA (Fig. 2 and Fig. 3A). To identify individual SB-ON species hybridized with tRNA, the bands, corresponding to tRNA-bound oligonucleotides were cut off the gel, the bound oligonucleotides were eluted from the gel and analyzed by polyacrylamide gel electrophoresis under denaturing conditions (Fig. 4B). Two major products with the length 13-mer and 12-mer, corresponding to SB-ON-B, SB-ON-C and one faint product, corresponding to oligonucleotide one base shorter then C, were selected at each SB-ONs mixture concentration. No other minor hybridization products have been observed.

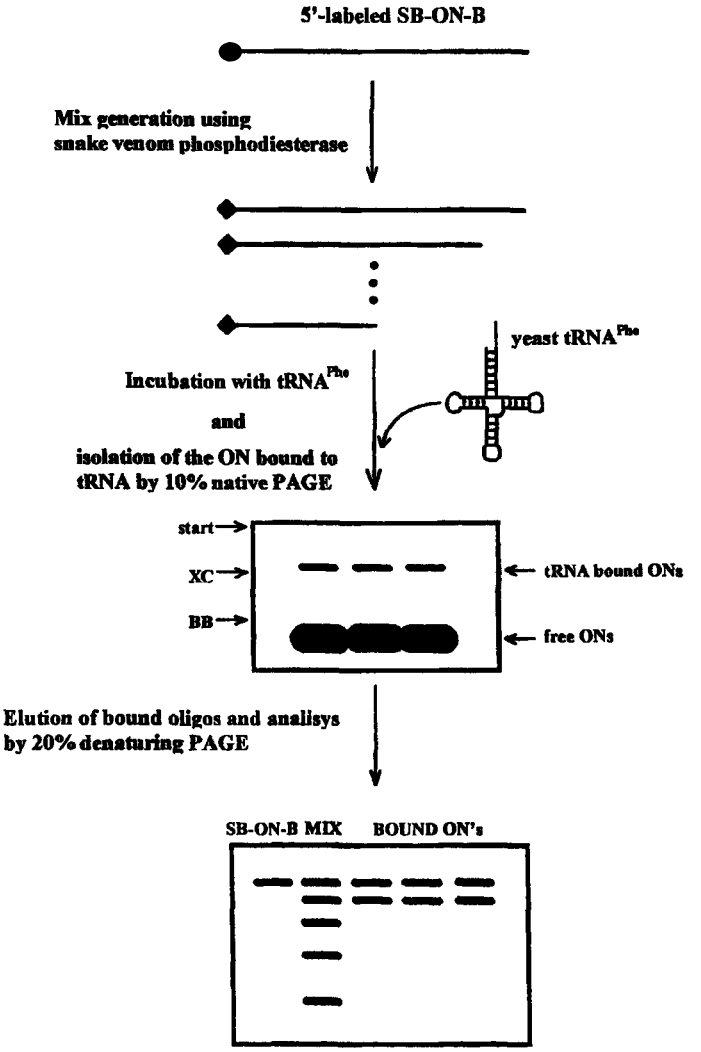


Fig. 4A. Scheme representing the selection experiment

All three selected SB-ONs (two major and one faint) share the same complementary site in tRNA^{Phe}, comprising the $ACCA_{OH}$-sequence and the acceptor stem, but differ in the number of bases complementary to the T-stem of tRNA (2, 1, and no bases for oligonucleotides SB-ON-B, SB-ON-C, and SB-ON-(C-1), respectively). Binding to the terminal $ACCA_{OH}$-sequence initiates hybridization and is the crucial event for the overall hybridization process. Pairing with tRNA bases in the T-stem seems to be needed for efficient binding, unfolding of the 3'-end region of tRNA, and extended duplex formation. Non-modified oligonucleotides shorter

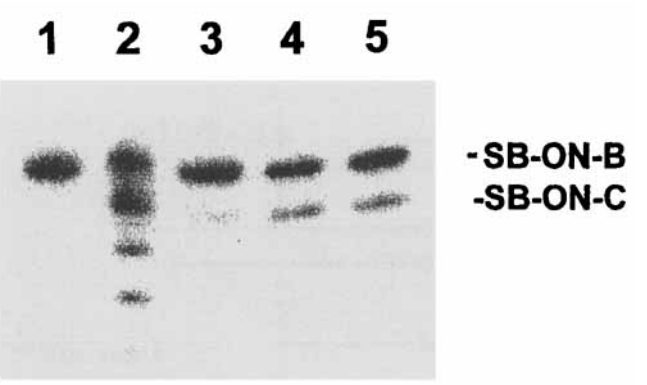


FIG. 4B. Experimental identification of strong binding oligonucleotides capable of binding to the 3'-part of $tRNA^{Phe}$ by electrophoresis in 20% polyacrilamide/ 8M urea gel. Oligonucleotide set was obtained by partial hydrolysis of 5'-[^{32}P]-SB-ON-B using snake venom phosphodiesterase. Lane 1 - SB-ON B; lane 2 SB-ON B hydrolized by snake venom phosphodiesterase; lanes 3,4,5 oligonucleotides extracted from the complex with tRNA after incubation with oligonucleotide mixture at total oligonucleotide concentration 20, 80, 240 μM, respectively.

than ON-C did not bind to $tRNA^{Phe}$ (unpublished data) and ON-C itself demonstrates rather poor binding with tRNA (see Fig. 3).

Altogether, the presented results evidence that incorporation of strongly binding bases in oligonucleotides improves their hybridization properties by stabilizing heteroduplexes with RNA and without changing the principles and fine mechanism of oligonucleotide-RNA hybridization. The benefit in hybridization efficiency depends on the structure of the oligonucleotide binding site and, in those cases, when low heretoduplex stability is crucial for effective hybridization, the benefit in equilibrium association constant for SB-ONs is maximal.

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