Hybridization of antisense oligonucleotides with the 3′ part of tRNA^{Phe}

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Received 21 December 1998

Abstract The interaction of antisense oligodeoxyribonucleotides with yeast tRNA^{Phe} was investigated. 14–15-mers complementary to the 3′-terminal sequence including the ACCA end bind to the tRNA under physiological conditions. At low oligonucleotide concentrations the binding occurs at the unique complementary site. At higher oligonucleotide concentrations, the second oligonucleotide molecule binds to the complex due to non-perfect duplex formation in the T-loop stabilized by stacking between the two bound oligonucleotides. In these complexes the acceptor stem is open and the 5′-terminal sequence of the tRNA is accessible for binding of a complementary oligonucleotide. The results prove that the efficient binding of oligonucleotides to the 3′-terminal sequence of the tRNA occurs through initial binding to the single-stranded sequence ACCA followed by invasion in the acceptor stem and strand displacement.

Key words: RNA structure; Unfolding; Complementary oligonucleotide; Strand displacement

1. Introduction

Antisense oligonucleotides (ONs) complementary to specific RNAs are used for controlling the expression of specific genes and are considered potential therapeutic agents [1]. The identification of optimal target nucleotide sequences for antisense ONs in RNAs represents a problem because under physiological conditions RNAs are folded in specific structures built of hairpins without extended single-stranded regions. Experimental studies indicate that in some cases ONs can bind to folded RNAs by invading certain elements of the RNA structure. Thus, it was found that complementary ONs can bind to some sequences in yeast tRNA^{Phe} in spite of the tight folding of this molecule [2,3]. Particularly efficient binding was observed with the 3′-terminal part of this RNA [4,5]. We investigated the interaction of ONs with this part of tRNA^{Phe} using the gel mobility shift assay and RNase H. Our results indicate that complementary ONs can easily invade the hairpin structure under physiological conditions, if a short single-stranded sequence in the target site is available to initiate the strand displacement process.

2. Materials and methods

ONs TGGTGCGAATTCTGT (1D), TGGTGCGAATTCTG (1A), GCGAATTCTGT (1H) and CTGAGCTAAATC (3A) (further referred to as oligos 1A, 1D, 1H and 3A, respectively) were synthesized by standard phosphoramidite chemistry and purified by ion-exchange and reverse-phase HPLC. The ONs were homogeneous as assayed by 20% PAGE in denaturing conditions followed by staining with Stains-All [6].

Yeast tRNA^{Phe} was a generous gift of Prof. G. Keith (IBMC, Strasbourg, France). T4 RNA ligase was purchased from Boehringer (Mannheim, Germany), 5′-[^{32}P]pCp with a specific activity of >4000 Ci/mmol was from BIOSAN (Russia). Yeast tRNA^{Phe} was labelled at the 3′-terminus with 5′-[^{32}P]pCp and T4 RNA ligase as described in [7].

2.1. Hybridization of tRNA^{Phe} with ONs

Hybridization of ONs to the tRNA^{Phe} was analyzed by a gel mobility shift assay [8]. Prior to hybridization, 3′-[^{32}P]tRNA (final concentration in the mixture 5×10^{-7} M) was heated at 90°C for 1 min, then cooled down and incubated at 20°C for 10 min. The 5×hybridization buffer (1×: 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 solutions ranging in concentration from 1×10^{-6} M to 1×10^{-3} M were added and the final mixtures (the 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 cyanole) 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 for 6 h at 350 V. To get quantitative data, the gels were dried, radioactive bands were cut out of the gel and their radioactivity was determined by Cerenkov counting.

2.2. Probing tRNA-ON complexes with ribonuclease H (RNase H)

Hybridization reactions were prepared and incubated as described above with one or two ONs in excess over tRNA. 1–1.5 U of RNase H (Promega) were added to the mixtures in buffer, supplemented with 2 mM DTE and incubated for 2 h at 20°C [9]. The reactions were quenched by precipitation of tRNA with 150 ml 2% solution of lithium perchlorate in acetone. RNA was collected by centrifugation and dissolved in the loading buffer (6 M urea, 0.025% bromophenol blue, 0.025% xylene cyanole) and the products were analyzed by electrophoresis in denaturing 12% PAG. An alkaline ladder and partial T1 RNase digest [10] were run in parallel with the RNase H cleavage products, to identify the cleavage sites.

3. Results

Two of the tested ONs, 1D and 1A (15- and 14-mers), are complementary to the sequences 62–76 and 63–76 at the 3′ end of tRNA^{Phe}, respectively. ON 1H is complementary to the sequence 62–72, lacking complementarity to the terminal ACCA fragment. ON 3A is complementary to the sequence 4–15 close to the 5′ end of the tRNA (Fig. 1).

The gel mobility shift assay was used to follow the hybridization of ONs with the tRNA at 20°C. It was found that ONs 3A and 1H do not bind to the tRNA in the concentration range 10–500 μM (gel shift primary data not shown). ONs 1D and 1A, at a concentration of 5×10^{-5} M, efficiently bind to the tRNA, giving complexes with a 75–85% yield. The results of the gel mobility shift assay are shown in Fig. 2A,B for the ONs 1D and 1A, respectively. Fig. 2C shows a secondary plot of the primary data of Fig. 2A,B obtained by measuring the intensity of the bands, corresponding to free tRNA, complex 1 and complex 2.

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PII: S0014-5793(99)00063-0

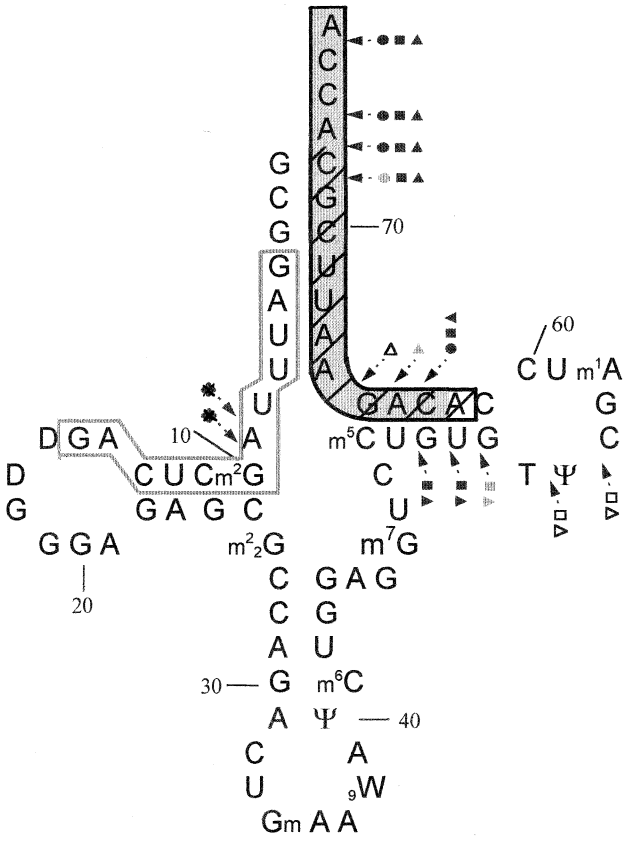

Fig. 1. Cloverleaf structure of yeast $tRNA^{Phe}$. The sequences complementary to ONs 1A, 1D, 1H and 3A are indicated: solid lines for ON 1D (black) and ON 3A (gray), gray shadow for ON 1A and black hatching for ON 1H. Circles and squares indicate the position of $tRNA^{Phe}$ cleavage by RNase H in the complex 1D-tRNA (circles, low ON 1D concentration 5×10^{-5} M; squares, high ON 1D concentration 5×10^{-4} M), triangles indicate ON 1A-tRNA (ON 1A concentration 5×10^{-5} M) and asterisks indicate ON 3A-tRNA in the presence of 1×10^{-4} M ON 1D (tertiary complex). Filled figures indicate a strong, half-filled a moderate and open figures a weak RNase H cleavage.

The concentration dependences of the complex formation by the ONs 1D and 1A with the tRNA are similar (Fig. 2C) except for the oligo 1A-$tRNA^{Phe}$ complex that is formed at lower ON concentrations as compared with 1D. An unexpected feature of the tRNA complexing with ONs 1A and 1D was the formation of two types of complexes, well-resolved by native PAGE, in the ON concentration-dependent manner. Complex 1 with higher electrophoretic mobility is formed at low ON concentrations. At high ON concentrations, accumulation of the slowly migrating complex 2 occurs. The second complex is formed more efficiently with ON 1A, the plateau level of complex 2 is achieved at a concentration of 1A of 100 μM. For 1D, the plateau level is achieved at an ON concentration over 500 μM. ON 1A hybridizes to $tRNA^{Phe}$ more efficiently than the longer ON 1D (85% and 75% of the tRNA is complexed under similar conditions with 1A and 1D, respectively), which contradicts the expected duplex stability.

The results suggest that an additional site for binding of ON 1A or 1D appears in the tRNA after binding of the first complementary ON molecule. Context analysis has revealed two additional potential binding sites, where the ONs 1D and 1A can form non-perfect complexes: 48–62, where six G-C and three A-T base pairs with 1A or 1D can be formed, and 1–15, where five G-C and three A-T base pairs can be formed. To identify the additional ON binding site, the tRNA-ON complexes were probed with RNase H (Fig. 3). The RNase H cleavage sites for the three ONs in different conditions are summarized in Fig. 1.

The results of RNase H probing are consistent with the data of the gel mobility shift assays and confirm our suggestion that the complexes 1 and 2 are formed consecutively. From the data presented in Fig. 3 (lanes 1–4), it is seen that the cleavage pattern depends on the ON 1D concentration. In the concentration range corresponding to the first complex formation (Fig. 2A) the main cuts are observed at positions 71, 72, 73 and 75, where the extended duplex should be formed. Increasing the 1D concentration results in the appearance of two new RNase H cuts at positions 50 and 51 and then at positions 52, 53, 55 and 63. Apparently these cuts reflect the formation of complex 2. These data support that a second ON binds to the sequence including positions 50–54 of $tRNA^{Phe}$ to yield complex 2. Similar results are obtained with ON 1A. The data are summarized in Fig. 1 (the primary data are not shown). The only difference was that the complex formation occurred at a lower ON concentration.

In the unfolded acceptor stem of the tRNA bound to 1D, the 5′-terminal sequence becomes available for the complementary ON (Fig. 3 lanes 9–12, lanes 5–8 for ON 3A binding). In the ternary complex of $tRNA^{Phe}$, 1D and 3A RNase H strongly cuts at position U8, this shows that oligo 3A binds to its complement in the presence of oligo 1D. The cut at position U8, even at the lowest concentration of 3A used (1×10^{-5} M), is the strongest, reflecting the effective pairing of 3A with the complementary sequence in the tRNA-1D complex.

4. Discussion

$tRNA^{Phe}$ has a compact structure in which only two short nucleotide sequences can be considered to be open and available for interaction with ONs: the anticodon sequence and the terminal ACCA sequence. Indeed, complementary short ONs can bind to these sequences although with a low affinity [11]. However, results of some studies suggest the possibility that ONs bind to extended sequences within the tRNA structure which can occur through disruption of the native tRNA structure. It is known that some tRNAs can serve as primers in the process of replication of retroviral genomes [12]. In this case reverse transcriptase assists in the opening of the tRNA structure and in the hybridization of the 3′ half of the tRNA molecule to the complementary RNA [13]. Binding of long synthetic ONs can be achieved with thermally unfolded tRNA molecules, where formation of such complexes is thermodynamically favorable [2,3]. Results of some studies indicate that ONs can invade some elements of the tRNA structure under physiological conditions, perhaps due to thermal 'breathing' of the structure, allowing the initiation of the process. In our experiments with ON conjugates complementary to the 3′-terminal sequence of the $tRNA^{Phe}$, we have observed spontaneous binding of the ONs to the tRNA [14]. In experiments with ON arrays, it was found that four of the 65 complementary 12-mers interact with $tRNA^{Phe}$ under conditions where the molecule should have a compact structure (4°C,

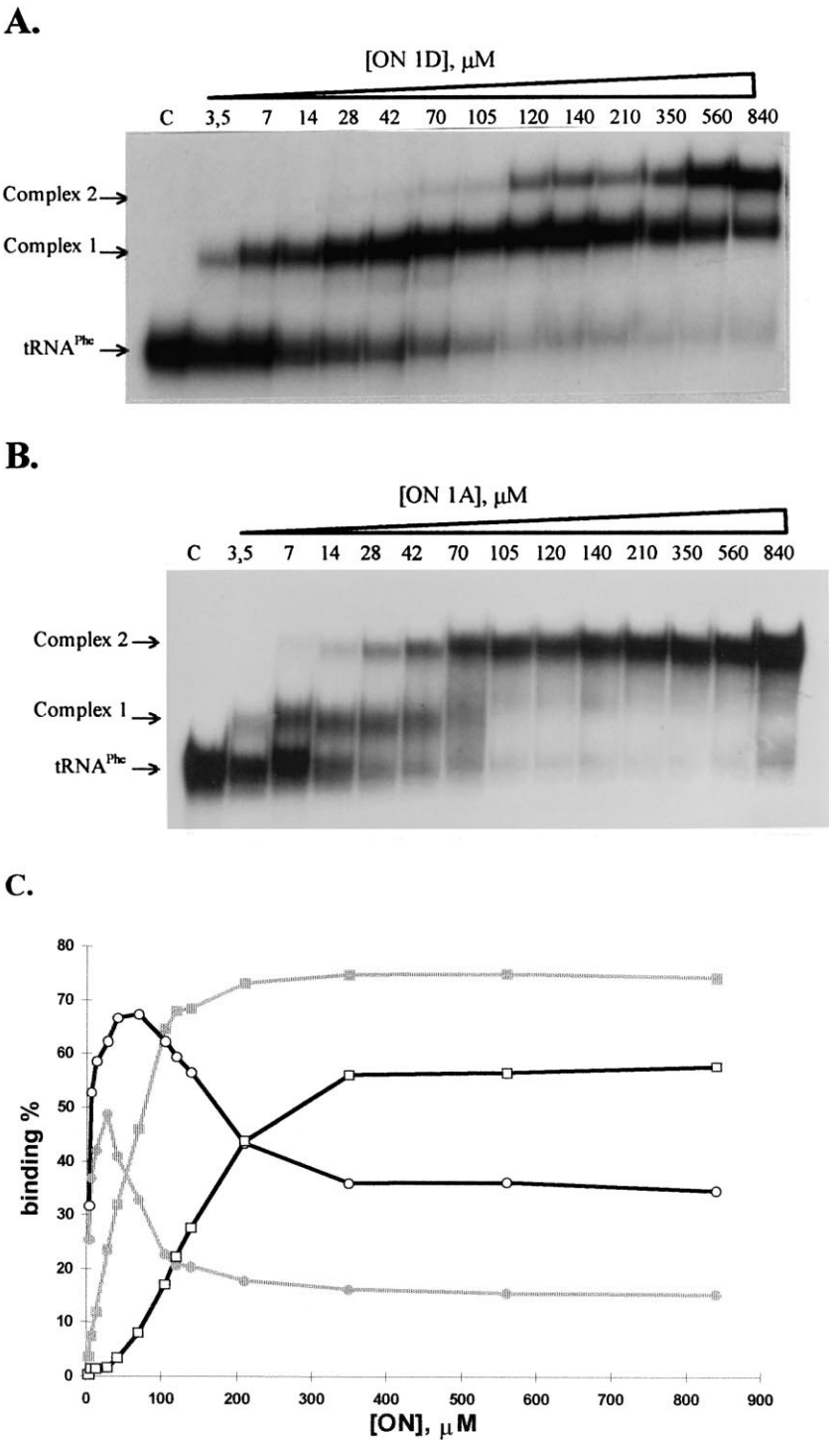

Fig. 2. Concentration dependence of the tRNA-ON 1D (A) and tRNA-1A (B) complex formation. Autoradiographs of the 10% native PAG. ON concentrations are shown at the top. On the left side, positions of free tRNA, complex 1 and complex 2 are indicated by arrows. C is a secondary plot of the data presented in A and B. The extent of the tRNA binding was determined as the ratio of the radioactivity measured in each specific band versus the total amount of radioactivity in the reaction mixture. Black curves are for ON 1D and gray curves for ON 1A binding. Circles indicate complex 1, squares indicate complex 2.

3.5 M tetramethylammonium chloride) [5]. The most stable complex was formed by the oligomer complementary to the 3′ end of the tRNA. The described results suggest that ONs can efficiently bind to the acceptor stem of the tRNA which is a typical hairpin structure with an adjacent single-stranded sequence. We investigated the interaction of $tRNA^{Phe}$ with ONs complementary to the sequences at the 3′ and 5′ ends of the molecule to obtain insight into the mechanism of the interaction and to characterize the structures of the complexes formed.

The target sequences for ON 3A and ON 1H are located within the stem region (Fig. 1). The target sequence for the

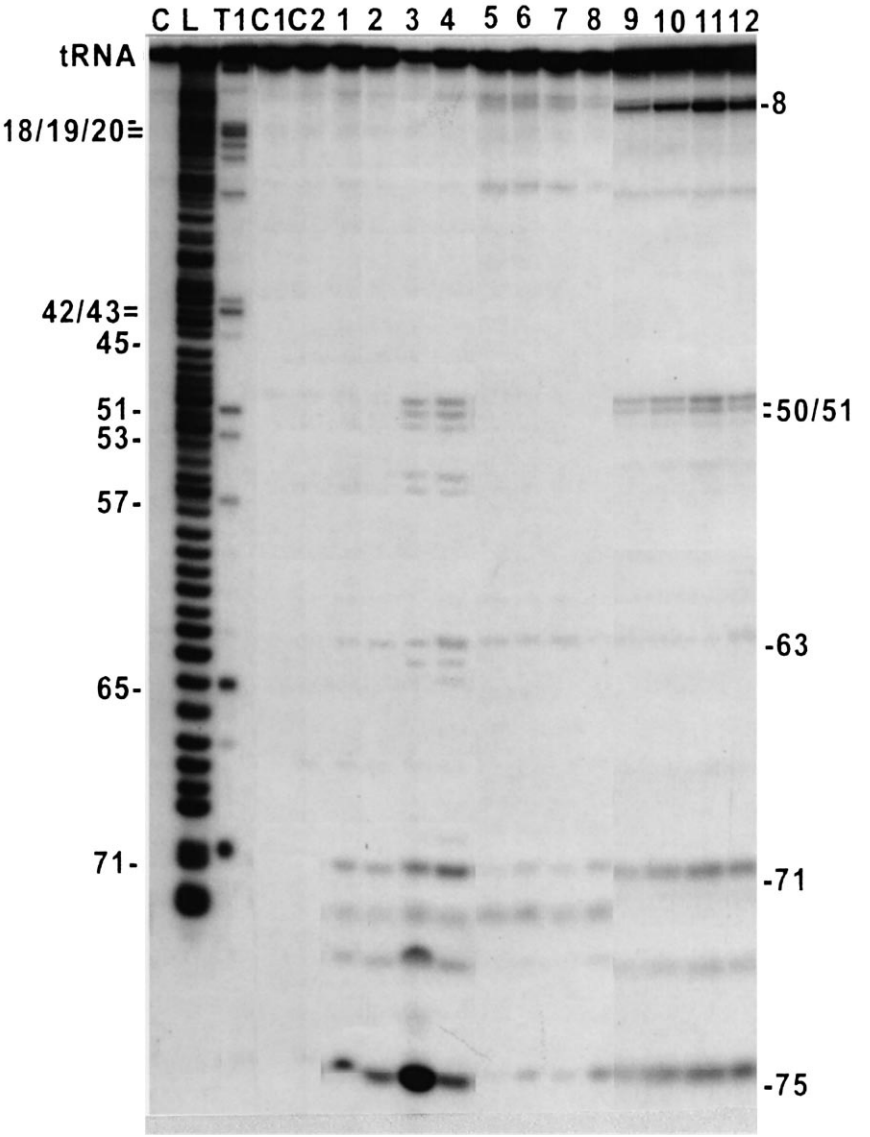

Fig. 3. Autoradiograph of denaturing PAGE of the probing tRNA complexed with ON 1D, 1A and 3A with RNase H. C = control tRNA, L = alkaline ladder, T1 = T1 RNase cleavage under denaturing conditions, C1 = tRNA+RNase H, C2 = tRNA+maximal 1D concentration. Lanes 1–4: 1D concentration 10 μM, 50 μM, 100 μM, 500 μM, respectively; lanes 5–8: ON 3A concentrations 10 μM, 50 μM, 100 μM, 500 μM, respectively; lanes 9–12, 3A, in the presence of 100 μM of 1D, at concentrations 10 μM, 30 μM, 50 μM, 70 μM, respectively.

ONs 1A and 1D includes the terminal single-stranded ACCA sequence, the sequence in the acceptor stem and in the T stem. The duplex stabilities for the ONs 3A, 1D and 1A, evaluated using the nearest neighbor method [15], were similar, but if the tRNA secondary structure was taken into account the duplex stabilities became (ΔG_{20}) −14.0, −11.6, −3.1 and −4.6 kcal/mol for 1D, 1A, 1H and 3A, respectively. These figures are in a good agreement with the experimental results: ON 3A and ON 1H did not form a complex with tRNA, whereas ON 1A and ON 1D efficiently bound to the $tRNA^{Phe}$. Binding of ON 1A with tRNA occurs even more efficiently than with ON 1D.

Apparently, the first step in the interaction of the ONs with tRNA is the initial binding with complementary single-stranded sequences. This process, similar to the nucleation during linear duplex formation [16], provides a possibility of invasion of ONs into the tRNA structure. Under the binding conditions in 0.2 M KCl, without Mg^{2+} ions, yeast $tRNA^{Phe}$ preserves a native-like folded structure [17]. The ONs 1A, 1D, 1H and 3A have structurally similar target sites within tRNA, but complements for the ONs 1A and 1D capable of binding to the tRNA include an open single-stranded ACCA sequence that provides effective nucleation between ONs and tRNA.

ON 1A is one residue shorter than 1D, but it binds to tRNA much more efficiently. Results of RNase H probing of the 1A-tRNA complex showed that even at low concentrations of the ON, two molecules of 1A bind to tRNA at the adjacent sequences 63–76 and 50–62. It seems likely that ON 1A first interacts with the 63–76 site by taking advantage of the effective nucleation. Binding of the second 1A molecule with the 50–62 sequence results in the formation of a contiguous duplex including the two stacked ONs. In the case of ON 1D, binding to the sequences 62–76 and 50–62 leads to interference at the contact of the ON ends which destabilizes the complex. This explains the decreased stability of the complex formed by 1D. Unfolding of the acceptor stem by 1A or

1D hybridization results in opening of the sequence complementary to 3A and leads to effective ON 3A binding to the opposite site of the tRNA molecule.

The obtained results demonstrate that targeting of nucleotide sequences in hairpin structures can be easily achieved by ONs complementary to the sequences in the hairpins and adjacent single-stranded open sequences.

Acknowledgements: This work was supported by Grant INTAS-RFBR 95-0653 and an Interdisciplinary Grant of the Siberian Division of the Russian Academy of Sciences.

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