FEBS Letters 392 (1996) 114–116 FEBS 17405

Hybridization of two oligodeoxynucleotides to both strands of an RNA hairpin structure increases the efficiency of RNA–DNA duplex formation

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Received 4 June 1996; revised version received 9 July 1996

Abstract Hybridization of two oligodeoxyribonucleotides (ON1 and ON2), complementary to opposite strands of the apical domain of *Escherichia coli* 4.5S RNA, was studied. ON1, complementary to bases 58–71, was not able to form a stable RNA–DNA hybrid whereas ON2, complementary to bases 38–53, was. Addition of both oligonucleotides at the same time resulted in the formation of a ternary complex permitting hybridization of ON1 and increasing hybridization of ON2. Under this condition, binary complexes of ON1 or ON2 with 4.5S RNA were not observed. The data demonstrate that hybridization of oligonucleotides to both strands of an RNA hairpin structure increases the efficiency of hybridization of either oligonucleotide.

Key words: Hybridization; 4.5S RNA; *Escherichia coli*; Oligodeoxynucleotide; RNA secondary structure

1. Introduction

The efficiency of antisense techniques depends on accessibility of the target RNA region for hybridization. Strong secondary and/or tertiary structures often prevent hybridization with oligonucleotides [1–3]. Therefore, studies on the dynamics of formation and structure of formed hybrids are necessary to develop effective hybridization methods.

4.5S RNA of *Escherichia coli* was chosen as a model since its apical domain contains a hairpin structure with several bulged bases [4]. Hairpins are the most frequently occurring RNA structure elements [5], and their hybridization with oligodeoxynucleotides is of significant interest [6]. The structure of 4.5S RNA was further refined by sequence alignment of RNAs found in signal recognition particles (SRP) considering base pairs for which comparative evidence was obtained [7,8]. Especially, secondary structures of apical domains are similar, even between bacteria and mammals [9,10], implicating common functional features. Secondly, 4.5S RNA supports protein synthesis [11,12] and forms together with the protein Ffh the SRP of *E. coli* [13]. Studies on the secondary structure of the apical domain will provide further information on the function of bacterial SRP as did mutation experiments [14]. Thirdly, hybridization studies are imperative towards site-directed modification of RNA by chemically reactive oligodeoxyribonucleotides as described in [15]. Although experiments have been performed to increase hybridization efficacy by using several [16] or adjacently located oligodeoxynucleotides [17], cooperative hybridization of both complementary strands of a hairpin structure has not been demonstrated.

Here we show that effectiveness of oligodeoxynucleotide hybridization to an imperfect RNA double helix depends on the number of bulged bases. Concerted attack of two oligonucleotides complementary to both strands of the proposed 4.5S RNA hairpin structure increase synergistically the hybridization efficiency of either oligodeoxynucleotide.

2. Materials and methods

2.1. Materials

4.5S RNA was overexpressed according to [4] using an *E. coli* strain transformed with pLH45 that was obtained from Dr. M.J. Fournier (University of Massachusetts). The RNA was labeled with [^{32}P]pCp and T4 RNA ligase as described [18]. Labeled RNA was purified by electrophoresis on 10% denaturating polyacrylamide gel in TBE buffer (0.089 M Tris-HCl, 0.089 M boric acid, 2 mM EDTA). The labeled RNA was eluted into 10 vol. of a buffer containing 0.3 M sodium acetate, pH 5.0, 0.1% SDS and 1 mM EDTA for 16 h, precipitated with 3 vol. of ethanol and dissolved in water. 1.06 nmol of 4.5S RNA correspond to 1 absorbtion unit at 260 nm. Oligodeoxyribonucleotides, ON1 (5′-GCCTTGGCTGCTTCp) and ON2 (5′-pCGGACCTGACCTGGTA) complementary to residues 58–71 and 38–53 of 4.5S RNA, respectively, were chemically synthesized.

2.2. Hybridization and electrophoresis of complexes

Oligonucleotides ON1 or ON2 or both were hybridized to 2 pmol labeled 4.5S RNA in a final volume of 2 μl containing buffer A (100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 40 mM Tris-HCl, pH 7.5) for 1 h at 25°C. Thereafter, 2 μl of loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF in TBE) was added and aliquots of 1.5 μl were separated on 10% polyacrylamide gel in TBE at 20 V/cm and room temperature. Electrophoresis was stopped when the xylene cyanol band reached the bottom of the gel. Gels were fixed, stained in 50% formamide and 0.05% 'Stains all' (Fluka) and dried. Radioactive bands were quantified using a phosphorimager (Raytest, Sprockhövel, Germany).

3. Results and discussion

The secondary structure model of 4.5S RNA proposed by [9,10] includes an apical domain, shown in Fig. 1, composed of a terminal tetraloop, short duplexes and two unpaired regions. The opposite strands are partly homologous and differ in the number of bulged bases. To study the proposed structure, oligodeoxyribonucleotides, ON1 (a 14-mer) and ON2 (a 16-mer), complementary to bases 58–71 and 38–53, respectively, were used for hybridization experiments. Enthalpies of both expected hybrids, calculated according to [19] should be similar and both duplexes are expected to melt at about 45°C. Considering the proposed secondary structure model, eight bulged bases are included within the structure formed by ON2 and 4.5S RNA. In comparison, only five bulged bases

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Abbreviations: ON, oligodeoxynucleotide; SRP, signal recognition particle

PII S0014-5793(96)00798-3

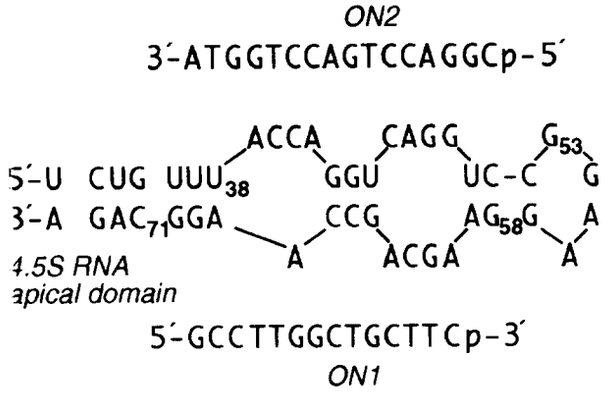

Fig. 1. The apical domain of 4.5S RNA as proposed by [9,10]. Two antisense oligodeoxynucleotides ON1 and ON2, complementary to bases 58–71 and 38–53, respectively, were used for hybridization experiments.

become engaged within the structure formed by ON1 and 4.5S RNA suggesting a lower probability for ON1 to hybridize. Indeed, ON1 was, as expected, almost unable to form a stable binary complex (Fig. 2A, lanes 7–11) as shown by analysis of gel mobility shifts. In comparison, ON2 formed a stable binary complex (Fig. 2A, lanes 2–6), although, 100-fold concentrations of oligomer were required to achieve a nearly complete hybrid formation (Fig. 2A, lane 4). Evidence was obtained that the minor band observed below the main form of binary complex may represent a secondary structure variant — rather than binding of ON2 to another sequence within 4.5S RNA — since crosslinking between 5'-activated ON2 and 4.5S RNA exclusively involves G_{53} (A. Malygin, in preparation).

Since ON1 and ON2 are not complementary to each other, they were applied simultaneously to study formation of a ternary complex. Remarkably, when ON1 and ON2 were employed in equimolar concentrations, the extent of ternary complex formation of both was increased in comparison to the amounts of binary complexes formed at the same oligonucleotide concentrations (Fig. 2B). Besides the ternary complex only traces of binary complexes were observed (compare Fig. 3). Binary complex formation was not increased even if all three components were used in an equal concentration of 10^{-6} M (data not shown).

The improved formation of the ternary complex may proceed via a stepwise reaction. Complex formation starts with hairpin melting and hybridization of one of the oligonucleotides — preferably ON2 — to 4.5S RNA. Thereby, the opposite strand of the hairpin would become available for hybridization with the second oligonucleotide, ON1. At this stage, the extent of ternary complex formation should coincide with the extent of ON2 hybridization at the same concentration. The observed additional increase in hybridization can be explained, considering that the binary complex is converted into the ternary complex, and additional binary complex is formed according to the mass-action law. In essence, the higher enthalpy of ternary complex formation induces an increased formation of binary and consequently, ternary complex.

In further experiments we determined the contribution of each oligodeoxynucleotide to the ternary complex formation. For this purpose the concentration of one oligonucleotide was kept constant and that of the other was increased (Fig. 3). The fractions of 4.5S RNA incorporated into the ternary complex were quantitated and the percentage of complex formation are given in Figs. 4 and 5. ON2 dramatically increased the efficiency of ON1 hybridization already at a 2-fold excess over 4.5S RNA (Fig. 4, ■) whereas no hybridization was recognizable in the absence of ON2 (Fig. 2, lanes 7–11 and Fig. 4, ○). The stimulatory effect of ON1 on hybridization of ON2 in the ternary complex was less pronounced (Fig. 5) since ON2 was already able to hybridize to 4.5S RNA on its own (Fig. 2, lanes 3–6 and Fig. 5, □). Data presented in Figs. 4 and 5 show that conversion of 4.5S RNA into a ternary complex can be optimized and that a 10-fold molar excess of ON2 and ON1 over 4.5S RNA is sufficient to convert more than 90% of 4.5S RNA into the ternary complex. The formation of two RNA–DNA duplex structures, involving nucleotides 38–53 and 58–

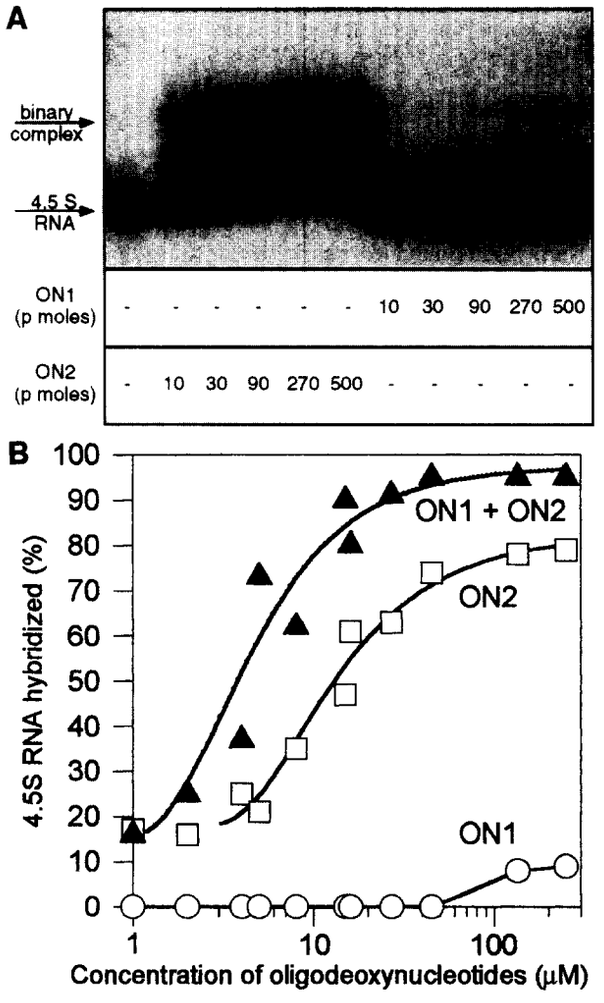

Fig. 2. Hybridization of oligodeoxynucleotides ON1 and ON2 to 4.5S RNA. (A) A typical gel mobility shift assay shows hybridization of ON2 (lanes 2–6) or lacking hybridization of ON1 (lanes 7–11) to ^{32}P-labeled 4.5S RNA. Lane 1 represents 4.5S RNA without additions as a control. Hybridization of 2 pmol 4.5S RNA were performed as described in Section 2. (B) Quantification of labeled 4.5S RNA in (A) shows that binary complex formation between 4.5S RNA and ON2 (□) needed a hundred-fold excess of ON2. Only traces of binary complex were observed with ON1 (○). Binding of either oligodeoxynucleotide — both at the indicated concentrations — is increased by formation of a ternary complex between 4.5S RNA, ON1 and ON2 (▲).

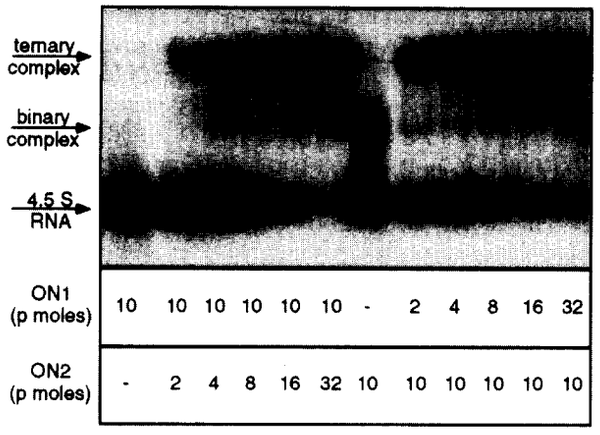

Fig. 3. Formation of a ternary complex of 4.5S RNA, ON1 and ON2 is shown by a typical gel mobility shift assay. The amount of 4.5S RNA was 2 pmol and amounts of ON1 (lanes 2–6) or ON2 (lanes 7–12) were kept at 10 pmol. The concentration of the second oligonucleotide was increased as indicated. Formation of binary complex between 4.5S RNA and ON2 is shown for comparison in lane 7.

71 of 4.5S RNA, within the ternary complex was confirmed by crosslinking experiments (A. Malygin et al., in preparation).

Thus, results obtained clearly demonstrate that hybridization of two antisense oligonucleotides complementary to both strands of an RNA hairpin structure increases the efficiency of RNA–DNA hybrid formation. By this means, hybridization of oligonucleotides that are unable to form a stable duplex by themselves, can be induced. This technique should improve functions of antisense therapeutics and ribozymes. Moreover, the method provides information on RNA structure and may be useful in investigating RNA secondary structure.

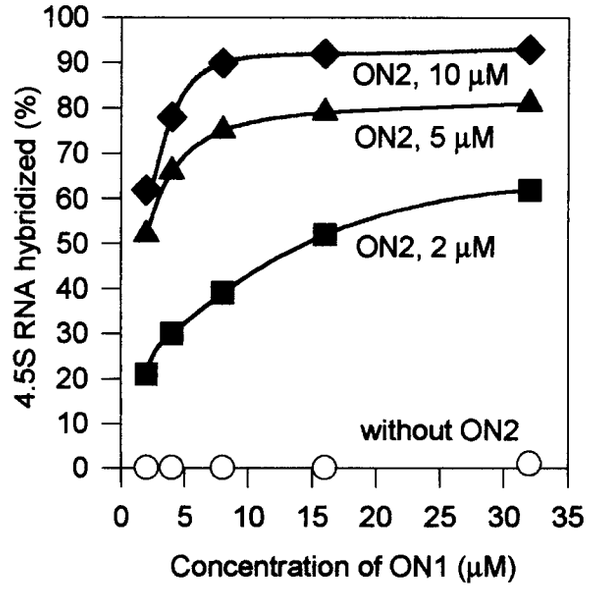

Fig. 4. Comparison of ternary complex formation at concentrations of ON2 of 2×10^{-6} M (■), 5×10^{-6} M (▲) or 10^{-5} M (◆). The concentration of 4.5S RNA was 10^{-6} M and concentrations of ON1 are indicated on the abscissa. For comparison, binary complex formation in the absence of ON2 is shown (○).

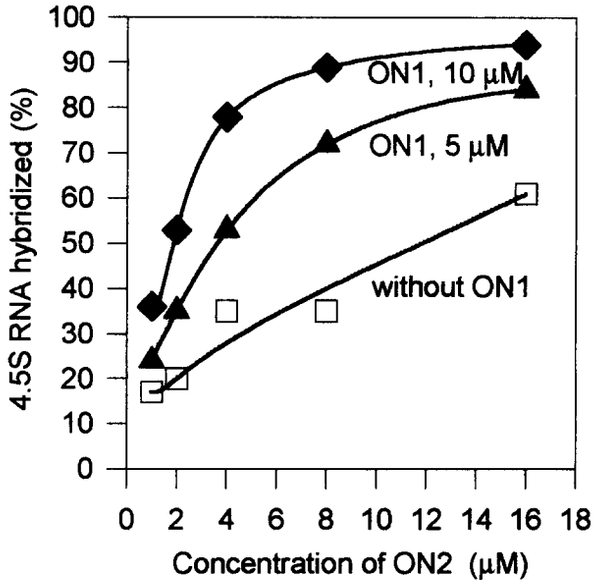

Fig. 5. Comparison of ternary complex formation at concentrations of ON1 of 5×10^{-6} M (▲) or 10^{-5} M (◆). The concentration of 4.5S RNA was 10^{-6} M and ON2 concentrations are indicated on the abscissa. For comparison binary complex formation in the absence of ON1 is shown (□).

Acknowledgements: We thank Dr. M.J. Fournier for providing us with 4.5S RNA and an overexpressing *E. coli* strain. The cooperative studies were generously supported by Volkswagen-Stiftung.

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