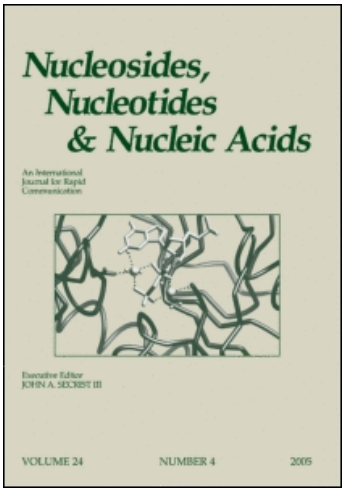

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Interaction of Escherichia Coli Ribonuclease H With Hybrid Duplexes Containing 2'-Deoxyxylotrymidine, 2'-Deoxy-2' Fluorouridine or Alpha-Thymidine

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INTERACTION OF ESCHERICHIA COLI RIBONUCLEASE H WITH HYBRID DUPLEXES CONTAINING 2'-DEOXYXYLOTHYMIDINE, 2'-DEOXY-2' FLUOROURIDINE OR ALPHA-THYMIDINE

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ABSTRACT

Oligothymidilates with repeating inserts of 1-(β-D-2'-deoxy-2'-fluoropentafuranosyl) uracil(fU), 1-(β-D-2'-deoxy-*threo*-pentafuranosyl)thymine(xT)an d α-oligodeoxyribonucleotide $\alpha(G_2T_{12}G_2)$ were synthesized. Hybrid duplexes were obtained to study the physico-chemical properties (melting curves, CD-spectra) and the interaction with *E.coli* ribonuclease H. It was found that the modified hybrid duplex $prA_{18}/TT(fUTT)_6$ did not bind the enzyme, the modified hybrid duplex $prA_{18}/(xTTT)_6$ inhibited oligoriboadenylate cleavage by RNase H in hybrid duplexes $prA_{20}/(G_2T_{20})$ and prA_{14}/T_{16}as well as the modified hybrid duplex $prA_{14}/\alpha(G_2T_{12}G_2)$.

INTRODUCTION

The enzyme ribonuclease H (RNase H) catalyzes the hydrolysis of RNA involved in RNA/ DNA heteroduplex formation in the presence of specific divalent cations, such as Mg^{2+} or Mn^{2+}[1] ; this enzyme is widely present in various organisms and plays an important role in DNA replication [2]. Sequence-specific inhibition of gene expression by antisense oligonucleotides has been successfully employed for a variety of viral and cellular targets [3,4], it was proposed that RNA degradation in the presence of .

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complementary oligodeoxyribonucleotides is due to the action of RNase H [2,5]. Earlier it was shown that modified oligodeoxyribonucleotides with inserted alternating 2'-O-methylcytidine, 2'-deoxy-2'-fluoronucleosides or ribonucleosides dramatically changed the hydrolytic efficiency of *E.coli* RNase H and the oligonucleotide structure influences the position of cleavage within the phosphodiester chain of RNA [6-8]. It was found also that nuclease-resistant α DNA/ βRNA hybrids are inhibitors of RNase H [9].

To clarify the influence of modified inserts in oligonucleotide probes on efficiency of RNase H action we developed a new approach based on comparison of the hydrolytic efficiency towards both naturally occurring and chemically modified nucleic acid duplexes. We studied RNase H-catalyzed cleavage of oligoriboadenylates in heteroduplexes prA_{20}/G_2T_{20} (D.1) and prA_{14}/T_{16} (D.2) in the absence or the presence of modified hybrid duplexes: $prA_{14}/\alpha(G_2T_{12}G_2)$ or $prA_m/(ZTT)_n$, where Z is the modified nucleoside. This model system allowed to estimate the thermodynamic stability of the hybrid duplexes by UV- spectroscopy, to reveal the geometry alterations within the helix by CD-spectroscopy and to evaluate binding of modified hybrid duplexes with the enzyme.

MATERIALS AND METHODS

Ribonuclease H (EC 3.1.4.34) from *E.coli* (4.05 mg/ml) was a gift from Dr.S.Kanaya (Protein Engineering Research Institute, Osaka, Japan). All the salts used were from Merck, Fluka (Germany) and Reakhim (Russia), special-purity grade; the water was purified on a Liqui Pure Laboratory purification system (U.S.A.).

Oligodeoxyribonucleotides T_{16}; G_2T_{20}; $TT(fUTT)_6$ and $(xTTT)_6$ were synthesized with an automatic synthesizer (Applied Biosystem 380B, U.S.A.) and isolated by HPLC (Altex chromatograph, U.S.A.). Alpha-oligodeoxyribonucleotide $\alpha(G_2T_{12}G_2)$ was synthesized using previously published protocols [10]. The purity of the oligonucleotides estimated by HPLC was 99.5%. To obtain phosphoramidites of fU and xT we used protocols [11,12].

Oligoriboadenylate 5'-phosphates prA_m (m = 14, 18, 20) were obtained by enzymatic hydrolysis of polyadenylic acid (1mg/ml, Serva, Germany) with *E.coli* RNase H (2.5 nM) in the presence of T_{16} ($1.4x10^{-5}$M) in buffer: 0.02M tris-HCl, pH 7.9, 0.15M NaCl, 0.011M MgCl, 0.5mM DTT, 1mM EDTA. Oligoriboadenylates were isolated by ion-pair HPLC or by 15% PAAG electrophoresis.

Ion-pair HPLC was carried out on HPLC chromatograph (Waters, USA) equipped with an Armsorb C16 (7.5 μ) column (4x250 mm) in 50 mM potassium phosphate buffer, pH 7.0, containing 2mM tetrabutylammonium phosphate; a logarithmic acetonitrile concentration gradient from 5 to 40% (flow rate 1 ml/min) at 47° C was used for elution. The extent of rpA_m hydrolysis within the rpA_m/T_n heteroduplex was calculated as a ratio between the prA_m peak area and that of nonhydrolyzable T_n.

The oligonucleotide concentration was determined spectrophotometrically. The following values of the molar extinction coefficients (ϵ_{260}) of the nucleotides were used: prA and pA - 15400; pT- 9300; pU - 8800; pC - 7300 and pG - 11700. ϵ_{260} of the anomalous nucleotides (pxT, pαT pflU pαG) were taken equal to $\epsilon_{260}$of natural analogs. Solutions of heteroduplexes were prepared by mixing equimolar amounts of the components.

The temperature dependence of the UV absorption of the heteroduplexes with continuous raising of the temperature at a rate of 0.5 °C /min and the change in the optical density of the solution in the course of the reactions catalyzed by RNase H, were followed with a Hitachi Model 150-20 spectrophotometer (Japan), using ter-mostatically controlled quartz cuvettes with a path length of 10 mm. The increase in UV absorbance at 260 nm (A_t-A_0) was monitored, and A_0 immediately after the enzyme addition was compensated to zero.

Hydrolysis of the heteroduplexes by *E.coli* ribonuclease H was carried out in 500 μl 0.02 M Tris-HCl buffer, pH 7.9, containing 0.15M NaCl, 11 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM EDTA. The reaction mixture was incubated 10 min at 10°C prior to the addition of the enzyme. The reaction was initiated by adding 2μl RNase H (final concentration 2.5 nM in the assay buffer). The spectrophotometric method (SM) for continuous monitoring of optical density in the course of enzymatic reaction

was used. For determination of the kinetic parameters 5-6 different concentrations of the substrate varying over 20-1000 nM were used. The experiments were performed at least in triplicate, and the average values were calculated.

CD spectra from 320 to 220 nm were recorded at 21°C with a dichrograph Roussel-Jouan III (France) and at 8°C with a computer-driven AVIV 62 DS CD spectrometer calibrated with (1s)-(+)-10-camphorsulfonic acid (Aldrich) and equipped with a thermoelectrically controlled cell holder. The cell compartment was continuously purged with dry N_2. Digitized data obtained at every nanometer of wavelength were corrected for baseline and smoothed by a least-squares polynomial fit up to the third order. CD-spectra per mole of monomer were plotted as $\epsilon_L - \epsilon_R$ in units of liter x mol^{-1} x cm^{-1}.

RESULTS AND DISCUSSION

E.coli RNase H is an extensively studied enzyme: amino acid sequences and residues in catalytic center are known, it was shown that the three-dimensional structure of RNase H domain in human immunodeficiency virus-1 is similar to that of *E.coli* RNase H [13-15]. The approach developed in the present work using the chemically modified oligodeoxyribonucleotide strand in hybrid duplexes allowed to address an important feature of RNase H, namely substrate binding.

To study the influence of modification in the deoxy strand of hybrid duplexes on the interaction with *E.coli* RNase H, non-modified and modified oligothymidilates were synthesized (see Table 1). Oligoriboadenylate 5'-phosphates (prA_{14}, $pr A_{18}$, prA_{20}) were used as oligoribonucleotide strands in hybrid duplexes. The choice of modified oligonucleotides was based on our earleir research, showing that (i) oligonucleotides with inserted alternating 2'-deoxy-2'-fluoronucleosides prevent cleavage of the RNA strand in the site opposite to this modification [8]; (ii) α-DNA/β-RNA hybrid duplexes inhibit the RNase H activity [9]; (iii)inserts of xT in oligodeoxyribonucleotides alter the geometry of phosphodiester fragment and increase resistance to an exonucleolytic attack [12,16].

A kinetic analysis of enzymatic activity is useful to study the enzyme-substrate interactions. The kinetic parameters of *E.coli* RNase H have been analyzed using different substrates [17-19]. Nevertheless, hybrid duplexes in which a RNA strand is flanked with DNA [17] or 2'-O-methyl RNA [18] may not be suitable substrates because their structure differs from that of a natural substrate. A spectrophotometric assay suggested in the present work allows to estimate appoximate values of kinetic parameters, due to multiple potential cleavage sites present within the prA_{20}/G_2T_{20} (D.1) or prA_{14}/T_{16} (D.2) duplexes. However, the simplicity of this fast protocol (including a very simple substrate synthesis) makes it possible to compare kinetic parameters of the RNase H cleavage of a natural substrate (e.g., D.1 and D.2) in the presence of modified heteroduplexes. We compared the rate of hydrolysis of oligoriboadenylates in the non-modified hybrid duplexes D.1 and D.2 in the presence or absence of the modified hybrid duplexes D.3 - D.5. The main shortcoming of the common classical technique to determine the enzymatic activity (measurement of the radioactivity of the acid-soluble fraction, analysis of cleavage products by PAGE or agarose electrophoresis, HPLC-analysis) are lack of continuous monitoring and/or extensive probe preparation. To circumvent these drawbacks we developed an UV-spectroscopy assay to detect degradation in double-stranded hybrid by RNase H. This method of continuous monitoring of the enzymatic reaction offers a number of advantages, such as rapidity, high precision in determining enzyme activity and allows to estimate the kinetic parameters of the reaction. The spectrophotometric method is based on the fact that cleavage of the RNA strand in the RNA/DNA- duplex by RNase H results in an increase in the UV absorbance at 260 nm (A_{260}) due to fragmentation of the ribo strand and formation of single stranded oligonucleotides [20]. The melting temperature of hybrid duplexes D.1 -D5 in the hydrolysis buffer and hyperchromic effects (h) are shown in Table 1. The melting temperature of these duplexes are notably different due to variation in chain length and modified nucleoside residues incorporated into the oligonucleotide duplex. Of all duplexes tested , βRNA-αDNA hybrid duplex (D.5) had the highest thermal stability in accordance with the earlier report [9]. According to the melting curves (not shown),

TABLE I.

Structure and melting temperature(T_m) of hybrid duplexes. Duplex concentration is $0.3x10^{-4}$ M per mononucleotide (700 - 1000nM per duplex).

#,Duplex	Duplex structure	Abbreviation*	Tm, C°	h, %
D.1	5'-GGTTTTTTTTTTTTTTTTTTT 3'-AAAAAAAAAAAAAAAAAAAp	$prA_{20}/(G_2T_{20})$	39	22
D.2	5'-TTTTTTTTTTTTTTTT 3'-AAAAAAAAAAAAAAp	prA_{14}/T_{16}	22	21
D.3	5'-TTfUTTfUTTfUTTfUTTfUTTfUTTfUTT 3'-AA-AAA-AAA-AAA-AAA-AAA-Ap	$prA_{18}/TT(fUTT)_6$	36	15
D.4	5'-xTTTxTTTxTTTxTTTxTTTxTTTxTTT 3'- AAA-AAA-AAA-AAA-AAA-AAAp	$prA_{18}/(xTTT)_6$	26	23
D.5	5'- α(GGTTTTTTTTTTTTTGG) 5'- pAAAAAAAAAAAAAA	$prA_{14}/\alpha(G_2T_{12}G_2)$	52	21

* - in DNA-strand symbol "d" is omitted

all the duplexes tested were stable at temperatures below 15°C and we routinely carried out all RNA cleavage experiments at 10°C . For instance, the kinetic curves in Fig. 1, Panel A show increase of optical density at 260 nm in the course of the RNase H catalyzed cleavage of RNA within the heteroduplexe D.1. The initial rate (O.D./min) of this enzymatic reaction was determined by differentiating the linear starting sections of the kinetic curves. To support the view that an increase of the optical density was due to RNA hydrolysis and subsequent heteroduplex dissociation we analyzed the hydrolysis products by HPLC.

Aliquot of the reaction mixture were taken during the reaction. The results of the chromatographic analysis of the hydrolysis products of prA_{20} are shown in Fig.1.Panel B. Using the spectrophotometric assay we measured the dependence of the initial rate of the RNase H catalyzed reaction according to the concentration of hybrid duplexes D.1 and D.2. The reaction kinetics followed the Michaelis-Menten equation. The values of the maximum rate(V_{max})and the Michaelis constant (K_m) for prA_m hydrolysis were found from the Hanes plot [21] as exemplified for D.2 on Fig.2, panel

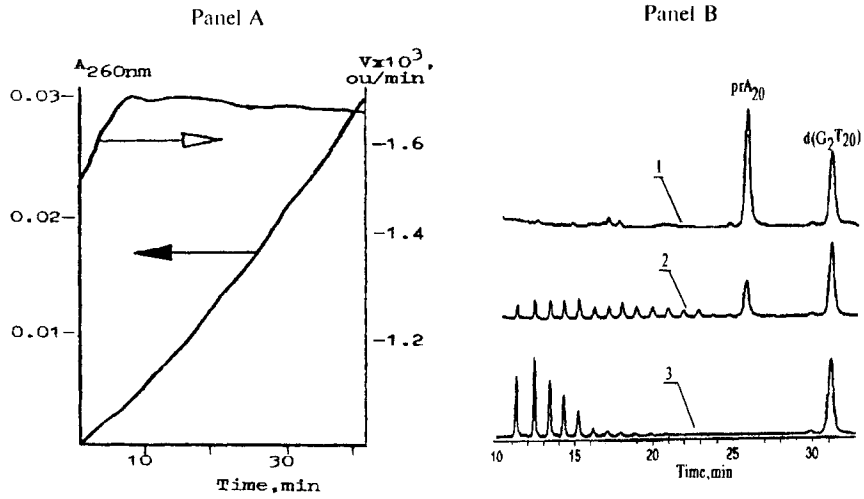

Fig. 1. Hydrolysis of RNA strand in hybrid duplex D.1(prA_{20}/G_2T_{20}) by *E.coli* RNaseH. Panel A: a kinetic curve (a filled arrow) of the change of the optical density at 260 nm in the course of enzymatic reaction, [S]=200 nM. The clear arrow shows a differential curve. Panel B: HPLC analysis of the reaction mixture; 1- at the start of incubation, 2- after 20 min of incubation, 3- after 60 min. of incubation.

A. The maximum rate was calculated using the proportionality coefficient b=17.1μM/O.D.[22] and was found to be 0.04 μM/min and 27 μM/min for D.1 and D.2, correspondingly . Data summarized in Table 2 suggest that affinity of a substrate increases in parallel to the chain length (cf. Km for D.1 and D.2). Similar correlation was found for heterogeneous oligomers differing in chain length [19].

Using spectrophotometry method we found that prA_{18} or prA_{14} in D.3 - D.5 were not cleaved by *E.coli* RNAse H . Next, we compared the influence of modified hybrid duplexes D.3 - D.5 on the efficacy of RNA cleavage in non-modified hybrid duplexes D.1and D.2. It was found that the addition of equimolar amount of D.3 did not change the rate of prA_{20} or prA_{14} cleavage in D.1 and D.2. But in the presence of duplexes D.4 or D.5 the rate of rpA_{14} or rpA_{20} cleavage decreased significantly. To calculate the K_I for duplexes D.4 and D.5 we found the rate of RNA hydrolysis in D.2 in the presence of different concentration of D.4 or D.5 using the Dixon equation [18]

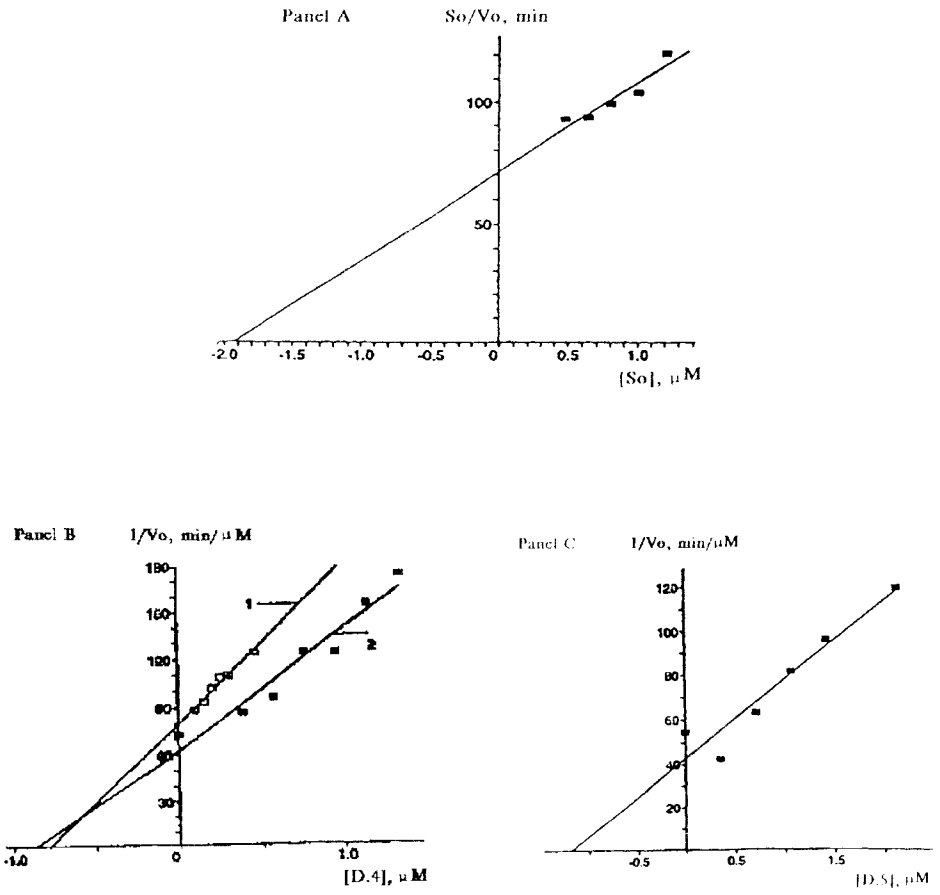

Fig. 2. Kinetics of RNA clevage by *E.coli* RNase H. Panel A: Hanes plot of the dependence of the initial rate of enzymatic hydrolysis of rpA_{14} in D.2 on the substrate concentration. Panel B: Dixon's plots of the dependence of the initial rate of enzymatic hydrolysis of rpA_{14} in D.2 on the D.4 concentration . 1-[D.2]=0.3 μM; 2-[D.2]=0.42 μM. Panel C: Dixon's plots of the dependence of the initial rate of enzymatic hydrolysis of rpA_{14} in D.2 on the D.5 concentration.

(see data in Fig.2 , Panels B-C and Table 2). The K_i's for D.4 and D.5 are similar, consequently D.4 inhibited the action of RNase H, similarly to D.5 whose inhibitory effect was shown earlier [9].

To estimate the overal structural alterations induced by modification in DNA-strands of D.3-D.5 we used CD spectroscopy. This method was used earlier to characterize structures of two hybrid duplexes, poly[r(A)/d(T)] and poly [r(A)/d(U)]

TABLE 2.

The affinity (Km) of D.1 and D.2 and the inhibition (Ki) of D.4 and D.5 related to *E.coli* RNase H.

Duplex	Abbreviation	Km,M	Ki,M
D.1	$prA_{20}/(G_2T_{20})$	$0.05x10^{6}$	-
D.2	$prA_{14}/(T_{16})$	$1.92x10^{-6}$	-
D.3	$prA_{18}/TT(fUTT)_6$	-	-
D.4	$prA_{18}/(xTTT)_6$	-	$0.67x10^{-6}$
D.5	$prA_{14}/\alpha(G_2T_{12}G_2)$	-	$0.51x10^{-6}$

[23] and to discriminate the conformational peculiarities of d(purine)/r(pyrimidine) and r(purine)/d(pyrimidine) hybrids [24]. CD spectra of modified hybrid duplexes werecompared to those of the natural RNase H substrates - prA_{20}/G_2T_{20} (D. 1) under RNA hydrolysis conditions (Fig.3, Panel A). It should be noted that the CD spectrum of D.1 has the characteristics that are intermediate between B-form and A-form helices. The duplexes D.5 and D.3 containing respectively anomer and fU residues in DNA strand differ significantly from D. 1 in CD amplitude and spectral shape at 300-255 nm, though D. 4 and D. 1 are quite similar. To study the changes in CD spectra induced by base pairing in the modified duplex D.4, we compared CD spectra of the double helix $rpA_{18}/(xTTT)_6$(Fig.3,B; curve "a") and the mean of the CD amplitudes of the single-stranded constituents (Fig.3,B; curve "d"). A slight difference in CD magnitudes found in spectra in question indicates that the strands of this hybrid have optical activities similar but not identical to those of the free single-stranded components. According to [25], all types of duplexes DNA/DNA, RNA/RNA, and hybrid DNA/RNA bind to RNase H, but only hybrid DNA/RNA duplex is altered in substrate-enzyme complex to be prepared for cleavage of RNA. On the other hand, some data indicate that the enzyme selects RNA/DNA substrate at the first stage of the

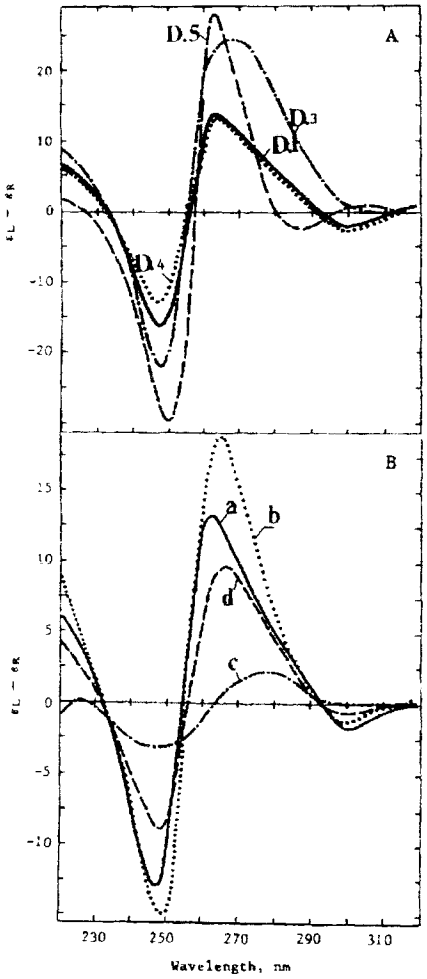

Fig.3. CD spectra of hybrid duplexes at 8°C in 0.02 M Tris-HCl buffer, pH 7.9, containing 150 mM NaCl, 11 mMMgCl$_2$, 0.5 mM dithiothreitol, 1 mM EDTA.
Panel A. Duplexes:
D.1, prA$_{20}$/(G$_2$T$_{20}$);
D.3, pA$_{18}$/TT(fUTT)$_6$;
D.4, pA$_{18}$/(xTTT)$_6$;
D.5, pA$_{14}$/α(G$_2$T$_{12}$G$_2$).
Panel B: a - modified duplex D.4, pA$_{18}$/d(xTTT)$_6$; corresponding components: b-prA$_{18}$; c-(xTTT)$_6$; d- average of the sum of the amplitudes of the single stranded components.

interaction [26] and our data confirms this suggestion. We have shown that RNase H is able to recognize and bind unusual sugar-modified oligonucleotides (see also [9]). Taking into account that heteroduplex D. 3 is not a competitor of the natural substrate of RNase H we suggested that it cannot bind to enzyme. The reason for that might be the replacement of a proton at 2'-position with electronegative fluoro atom and/or conformational changes in D.3 caused by such a replacement; the amount of C-3' endo(N) conformer is known to increase linearly with the electronegativity of the 2'-substituent [20]. In other words, introduction of fU-residues into oligodeoxyribonucleotides results in the change of the total geometry of D.3 to RNA/RNA-like structure (see[8]). Evidently, the rejection of this substrate occurs already at the stage of the enzyme-substrate complex formation (see also [26]). Although $rpA_{18}/(xTTT)_6$ (D.4) and $rpA_{14}/\alpha(G_2T_{12}G_2)$ (D.5) are not substrates for RNase H (see above and [26,27]) , they are able to inhibit considerably the RNA cleavage in substrate (D.1 and D.2). According to CD data, D.4 has a secondary structures quite similar to that of the nonmodified hybrid (Fig.3,Panel A). CD data confirm significant structural alterations in D.5 duplex compared to unmodified duplex. It is known that αDNA-βRNA duplexes adopt a right-hand helical form with parallel orientation of interacting chains [28].The adoption of an S-type sugar pucker for both strands and anti sugar-base orientation indicates that the αDNA-βRNA duplexes may form B-type helix [28]. Therefore, D.4 and D.5 are likely to associate with RNase H giving stable complexes. However, enzyme-substrate complexes of RNase H with both $rpA_{18}/(xTTT)_6$ and $rpA_{14}/\alpha(G_2T_{12}G_2)$ are not productive. We suggest that sugar modifications (inversion of hydroxyl function at C-3' atom or inversion of configuration at the anomer carbon atom) alter the geometry of phosphodiester fragment in DNA-strand and, as a consequence, in complementary RNA, so that the interactions between functional groups of the enzyme and the substrate in the active site are suboptimal. The resistance of arabino- [15], xylo- [15] and α-oligonucleotides [29] to PDE hydrolysis is consistent with this view.

Thus, D.4 and D.5 can be considered as competitors for the natural DNA/RNA substrates in their binding to the active site of RNase H inhibiting in this way the

enzymatic activity. Antisense technology is now a rapidly developing approach aimed to selectively affect gene expression. An effective antisense probe may require chemical modification of nucleotides which are important for: (i) stability to cellular nucleases,(ii) high incorporation of antisence oligonucleotides into cell,(iii) high affinity, sequence-specific interaction with target RNA, (iv) effective RNA cleavage by RNase H . The spectrophotometric assay of hydrolysis of prA_m/T_n duplex by *E.coli* RNase H in the presence of modified hybrid duplex is a simple approach to estimate the influence of modification in DNA strand on the interaction with RNase H.

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