The influence of oligonucleotide-effector on the selectivity of sequence specific modification of 16 S rRNA

M.A. Zenkova, O.S. Fedorova, A.S. Levina, S.V. Mamaev and G.G. Karpova

Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of USSR Academy of Sciences, 630090 Novosibirsk, USSR

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The influence of duplex stabilizing oligonucleotide-effector (oligonucleotide, carrying *N*-(2-hydroxyethyl)phenazinium residues on both ends), on selectivity of site-directed modification of *E. coli* 16 S rRNA (1542 nucleotides in length) under the conditions of its secondary structure stability was studied. The constant of cooperative binding of the reagent and the oligonucleotide-effector with 16 S rRNA was determined. The accuracy of modification was shown to double in the presence of 50 μM effector at 5 μM concentration of the reagent.

Sequence specific modification; Selectivity; Oligonucleotide-effector

1. INTRODUCTION

One of the key problems of the hybridization arrest of translation by antisense oligonucleotides both in vitro [1,2] and in vivo [3] is to find the ways to enhance the accuracy of directed influence of these oligonucleotides on the target.

Earlier site-directed modification of *E. coli* 16 S rRNA with 4-[*N*-methyl-*N*-(2-chloroethyl)amino]-benzylidene derivatives of oligodeoxynucleotides representing reactive analogs of antisense oligonucleotides was studied [4–7]. 16 S rRNA was used as a model of high molecular weight target with complex spatial structure. It was found that, under the conditions of 16 S rRNA secondary structure stability (ionic strength 0.37 M) and at reagents concentrations within the range of 5–30 μM [4], not only target sites were alkylated. Along with them, other sequences undergo modification, forming imperfect duplexes (duplexes with mismatches and dangling ends) with the reagent. The selectivity of modification could be improved, therefore, by excluding these imperfect complexes from the sphere of reaction.

Recently a principally new method to enhance selectivity of sequence-specific modification was proposed [8–10]. It is based on using oligonucleotide-effector (E) carrying *N*-(2-hydroxyethyl)-phenazinium residues working as an intercalator on one or both ends and complementary to the nucleic acid sequence adjacent to the reagent (X) binding site [10]. The accuracy of modification was improved due to the cooperativity of binding of the X and E with the target. This resulted in lengthening of the double-helix region formed by the reagent and effector with nucleic acid.

This study is the first attempt to analyze the use of the oligonucleotide-effector for enhancement of selectivity of site-directed alkylation of high molecular weight target (16 S rRNA). The alkylating agent (reagent or X) was 2′,3′-*O*-[4-*N*-methyl-*N*-(2-chloroethyl)amino]benzylidene derivative of d(pTTTGCTCCCC)rA. The effector was 3′,5′-diphenazinium derivative of p(dTAATCCTG)p.

Correspondence address: M.A. Zenkova, Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of USSR Academy of Sciences, 630090 Novosibirsk, USSR

Abbreviations: R, reagent, 2′,3′-*O*-[4-*N*-methyl-*N*-(2-chloroethyl)amino]benzylidene derivative of d(pTTTGCTCCCC)rA; Phn, *N*-(2-hydroxyethyl)phenazinium; PAAG, polyacrylamide gel

2. MATERIALS AND METHODS

RNase H was kindly donated by Dr N.V. Chichkova; plasmid pKK 3535 was a gift of Dr H.F. Noller; T4 RNA-ligase, RNase A, restriction enzymes *Xba*I, *Bam*HI, *Msp*I, *Hae*III, *Alu*I were purchased from NPO 'Ferment' (USSR); other reagents were from Fluka, Merck, Sigma and Serva.

Synthesis of the reagent (X), having either ^{32}P-labelled 5′-end of the oligonucleotide part or [^{14}C]-labelled reactive fragment, was performed according to the method described in [11].

Synthesis of E: Phn-p(dTAATCCTG)p-Phn was carried out in accordance with [8].

Isolation and preparation of 16 S rRNA for site-directed alkylation by such type of reagents are described in [5]. Modification of 16 S rRNA by X was performed in TMK_{350} buffer (50 mM Tris-HCl, pH 7.4; 20 mM $MgCl_2$; 0.35 M KCl) at RNA concentration of 0.5 μM for 30.3 h at 20°C. 50% of X was converted into ethyleneimmonium cation (the reactive intermediate) under these conditions. The initial concentration of the reagent being constant, the concentration of E varying. The reaction mixture contained 5 nmol of 16 S rRNA and was 10 μl in volume. The reaction was terminated by precipitation of RNA by 10 vols of 2% $LiClO_4$ in acetone. The alkylated 16 S rRNA was separated from the unreacted reagent by denaturing PAAG 6% electrophoresis (20 × 30 × 0.02 cm). The extent of modification was determined from the amount of the ^{32}P-label in the 16 S rRNA band.

The assays of selectivity of modification. 16 S rRNA was alkylated

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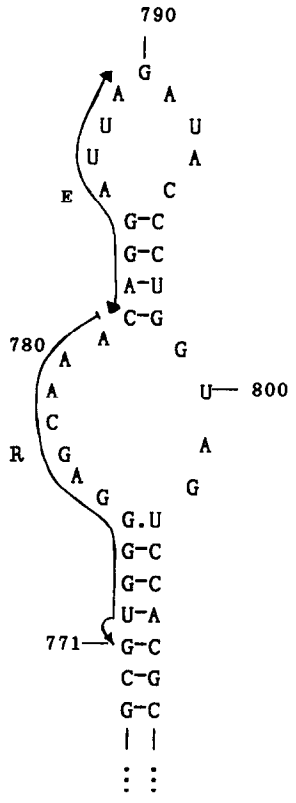
by ^{14}C-labelled X on preparative scale (150 pmol of RNA). The covalent adducts 16 S rRNA-X (the products of sequence specific modification of 16 S rRNA by X) were isolated according to [6]. RNase H hydrolysis of RNA-chain within the covalent adducts as well as identification of alkylated sites by blot-hybridization of RNA-fragments (obtained by RNase H hydrolysis of the covalent adducts) to the restriction fragments of pKK 3535 plasmid [12], are described in detail in [6,7].

3. RESULTS AND DISCUSSION

16 S rRNA *E. coli* was chosen as a model to test the oligonucleotide-effector (E) ability to improve the accuracy of sequence-specific modification of highly structured target. The 3′,5′-diphenazinium derivative of p(dTAATCCTG)p (E) was complementary to 16 S rRNA sequence 782–789 which was adjacent to the target sequence 771–781. Fig. 1 shows the structure of 16 S rRNA fragment including the sequences complementary to the X and E.

The nucleic acids modification by reactive oligonucleotide derivatives in the presence of E can be described by the following kinetic scheme:



R p(dTTTGCTCCCC)rA>CHRCl

>CHRCl= ^{14}CH–N(CH_2CH_2Cl)(CH_3)

Fig. 1. Structure of 16 S RNA domain comprising a full complementary copy of the reagent (X) and oligonucleotide-effector (E).

$$\begin{array}{ccccc} nPX + PXE & \xrightarrow{k_o} & nPI + PIE & \xrightarrow{k_1} & nPZ + PZE \\ \downarrow\uparrow & & k_{-1}\downarrow\uparrow k_1 & & \\ P + X + E & \xrightarrow{k_o} & P + I + E & \xrightarrow{k_2} & P + R + E \\ & & & & \rightleftharpoons nPR + PRE \end{array}$$

where P is target nucleic acid; X is reagent; E is oligonucleotide-effector; I is reactive ethyleneimmonium cation formed at the limiting step of the reaction; R is product of reagent hydrolysis in solution; PZ is the product of modification; PX, PE, PI, PR, PXE, PRE, PZE are complexes formed within this system; k_o, k_1, k_2, k_1, k_{-1} are the reaction rate constants shown in the scheme. Presumably, target P has a number of binding sites n for X (one of them is fully matched and others are imperfect) and a single site for E binding (this site is adjacent to the target region forming fully matched duplex with X). The rise of X concentration increases the amount of all n PX complexes that leads to the non-specific modification of the target; while the increase in E concentration should stimulate the formation of PXE complex only. Due to cooperative nature of X binding in PXE complex, the formation of PX complexes is less favourable and the level of modification in imperfect complexes should be lower, than that without effector.

Fig. 2 represents the extent of 16 S rRNA modification by X versus concentration of the E. We observed a rather small range of concentrations (Fig. 2, curves 1–5): 20–100 μM, at which the increase of E concentration led to a significant increase in the extent of modification (α_o). The largest increase of α_o (up to 0.32 and 0.40) was observed at X concentrations 0.5 μM and 1.5 μM, respectively, in the presence of E (Fig. 2, curves 1, 2). In the absence of E at the same X concentrations, α_o was equal to 0.06 and 0.10, respectively. When X concentration was increased up to 5 μM in the

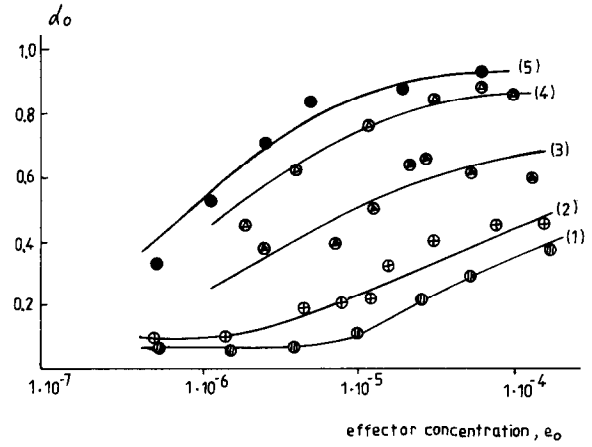


Fig. 2. The extent of 16 S rRNA modification (α_o) by X versus concentration of effector (e_o) at 20°C; 16 S rRNA concentration was equal to 0.5 μM, X concentrations were 1, 0.5 μM; 2, 1.5 μM; 3, 2.5 μM; 4, 4.0 μM; 5, 5.0 μM.

presence of E, the increase of α_o on the plateau reached 0.60 (Fig. 2, curve 5).

Based on the data of 16 S rRNA modification at 20°C in the presence of E, we calculated the constant of cooperative binding of X and E with RNA target (K_{XE}). K_{XE} was equal to $(8.4 \pm 3.5) \times 10^{10}$ M^{-2}. K_{XE} can be expressed as $K_{XE} = a \cdot K_X \cdot K_E$, where a is cooperativity factor; K_X and K_E are binding constants of X and E with 16 S rRNA. K_X value was determined in [4]. However, since K_E is unknown we could not calculate cooperativity factor a.

Earlier, it was shown that X could bind 16 S rRNA at 12 sites, forming (besides 771–781) imperfect duplexes with the reagent. Among 12 sites found for X, 3 (663–673, 442–452, 883–893) could form imperfect complexes which were of similar stability as the perfect one with site 771–781 [7]. In the separate experiments we assayed selectivity of 16 S rRNA modification in the presence and absence of E (see Table I). The measures of selectivity of sequence specific modification were chosen to be (i) relative extent of modification onto the selected site of 16 S rRNA (771–781; α_1/α_0; see Table I), and (ii) relative extent of modification within 3 most stable imperfect complexes and within the selected site (α_2/α_0). The resulting changes of selectivity of 16 S rRNA modification in the presence of E are given in Table I. It is seen that the changing of 16 S rRNA modification extent in the presence of E is due to the increase of modification extent at the selected site. In the absence of E, the modification at 771–781 amounts to about 20% of the overall amount whereas in the presence of E this value is more than doubled.

So, the attempts to improve accuracy of sequence-specific alkylation of high molecular weight RNA by using oligonucleotide-effectors carrying *N*-(2-hydroxyethyl)-phenazinium residues seem to be successful. Our data show that at a concentration of the reagent in the μM range, the application of 50 μM effector concentration brings about more than two-fold enhancement of selectivity of sequence-specific modification.

Table I

The effect of oligonucleotide-effector on the selectivity of modification of 16 S rRNA by p(dTTTGCTCCCC)rA > CHRCl

N	[R] (M)	[Ef] (M)	α_0[a]	Selectivity of sequence-specific modification			
				α_1[a]	α_1/α_0 (%)	α_2[a]	α_2/α_0 (%)
1	0.5×10^{-6}	5×10^{-5}	0.64	0.24	38.8	0.47	73
2			0.25	0.048	20.0	0.17	68
3	1.5×10^{-6}	5×10^{-5}	0.92	0.42	45.0	0.66	72
4			0.51	0.10	20.5	0.33	65
5	5.0×10^{-6}	5×10^{-5}	1.18	0.62	54.0	0.85	72
6			0.60	0.13	24.5	0.35	60

[a] α_0 = the extent of modification of the whole 16 S rRNA by R (mol X per mol of 16 S rRNA) is calculated from the amount of ^{14}C-label retained by RNA after incubation with the X and separation from the unreacted X by centrifugation in 3–20% sucrose density gradient in the presence of 0.5% SDS [5]; α_1 = extent of modification of the target sequence 771–781; α_2 = sum of extents of RNA modification within 3 sites (442–452, 663–673, 883–893) forming the most stable imperfect duplexes with the X and extent of RNA modification at the target site 771–781; α_1 and α_2 were determined using scanning data of the radioautogram of the blot with immobilized restriction fragments of rDNA after blot-hybridization to the RNase H hydrolysate of covalent adducts 16 S rRNA-X, washing and RNase A treatment of the blot as described in [7]

REFERENCES

[1] Agris, C.H., Blake, K.R., Miller, P.S., Reddy, M.P. and Ts'o, P.O.P. (1986) Biochemistry 25, 6268–6275.

[2] Agrawal, S., Ikeuchi, T., Sun, D., Sarin, P.S., Konopka, A., Maizel, J. and Zamecnik, P.C. (1989) Proc. Natl. Acad. Sci. USA 86, 7790–7794.

[3] Jaskulski, D., Kim de Riel, J., Merser, W.E., Calabretta, B. and Baserga, R. (1988) Science 240, 1544–1546.

[4] Gorshkova, I.I., Zenkova, M.A., Karpova, G.G., Levina, A.S. and Soloviev, V.V. (1986) Mol. Biol. 20, 1084–1096.

[5] Zenkova, M.A., Karpova, G.G. and Levina, A.S. (1987) Mol. Biol. 21, 1030–1036.

[6] Zenkova, M.A., Karpova, G.G. and Levina, A.S. (1989) Mol. Biol. 23, 1057–1066.

[7] Zenkova, M.A., Karpova, G.G., Levina, A.S., Mamaev, S.V. and Soloviev, V.V. (1990) Bioorg. Khim. 16, 788–800.

[8] Zarytova, V.F., Kutyavin, I.V., Silnikov, V.N. and Shishkin, G.V. (1986) Bioorg. Khim. 12, 911–920.

[9] Vlassov, V.V., Zarytova, V.F., Kutyavin, I.V., Mamaev, S.V. and Podyminogin, M.A. (1986) Nucleic Acids Res. 14, 4065–4076.

[10] Kutyavin, I.V., Podyminogin, M.A., Bazina, Yu.N., Fedorova, O.S., Knorre, D.G., Levina, A.S., Mamaev, S.V. and Zarytova, V.F. (1988) FEBS Lett. 238, 35–38.

[11] Ryte, V.K., Karpova, G.G. and Grineva, A.S. (1977) Bioorg. Khim. 3, 31–38.

[12] Brosius, J., Dull, T.J., Sleeter, D.D. and Noller, H.F. (1981) J. Mol. Biol. 148, 107–127.