SELECTIVE ALKYLATION OF POLY(A) TRACTS OF RNA INSIDE THE CELL WITH THE DERIVATIVE OF ETHYL ESTER OF OLIGOTHYMIDILATE BEARING 2-CHLOROETHYLAMINO GROUP

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1. Introduction

The method of the complementary addressed modification was proposed [1] and realised [2–4] by N. I. Grineva et al. to perform the selective labeling of single-stranded nucleic acids at the predetermined points. The methods were elaborated for the binding of the reactive 2-chloroethylamino group to either 3′- or 5′-end of oligonucleotides directing the reagent to the chosen region of nucleic acid [5,6]. The selective mutagenesis of T7 DNA has been described, based on the mRNA derivatives with 2-chloroethylamino groups attached to the guanine residues [7]. A similar idea was presented in [8].

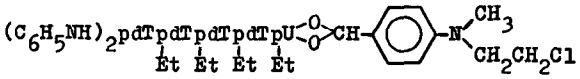
To employ complementary addressed modification in situ it is necessary to overcome at least two difficulties:

(i) The low ability of the hydrophilic oligonucleotide or polynucleotide derivatives to cross the outer cell membrane;

(ii) The sensitivity of the oligonucleotide moiety of the reagents of the above type to intracellular nucleases.

Esterification of the internucleotide phosphates with ethanol does not result in the loss of the ability of oligonucleotides to undergo Watson-Crick base-pairing [9,10]. At the same time these non-charged oligonucleotide analogs are sufficiently hydrophobic to penetrate through the cell membrane and exhibit rather high stability towards digestion by nucleases [11].

Here, we describe the alkylating pentanucleotide derivative $(C_6H_5NH)_2p(dTpEt)_4U$ with completely esterified internucleotide phosphates and with 2′,3′-*O*-4-(*N*-2-chloroethyl-*N*-methylamino)-benzylidene residue attached to the 3′-end of the molecule.



(I)

This reagent was demonstrated to penetrate efficiently into Krebs ascites tumor cells and to alkylate nucleic acids inside the cells with a strong preference towards poly(A) tracts of mRNA.

2. Materials and methods

Synthesis of tetraethyl ester of the 5′-bisanilidate of pentanucleotide $(C_6H_5NH)_2p(dTpEt)_4U$ was performed by E. A. Sheshegova and V. F. Zarytova by commonly-used methods and is described separately. The alkylating derivative [^{14}C]I was obtained by treatment of oligonucleotide with 4-(*N*-2-chloroethyl-*N*-methylamino)-[^{14}C]benzaldehyde in dry dimethyl formamide in the presence of 2,2-dimethoxypropane and CF_3COOH as in [12]. According to the ratio oligonucleotide:aldehyde, determined as in [13], the product was 92% pure. The molar extinction ϵ_{260}^{pH7} was 63×10^3 M^{-1} cm^{-1}. The solubility in water was near 40 μM.

[^{14}C]I (1.7–35.4 μM) was incubated for 36 days at 5°C with 0.17 mM poly(A) (given in nucleotide residues) in 0.01 M Tris–HCl (pH 7.5) containing 0.2 M NaCl and 0.01 M $MgCl_2$. Alkylated poly(A) was isolated by gel-filtration through Sephadex G-75 at 45°C. The extent of modification was determined by measuring the radioactivity of the poly(A) fraction and was expressed as mol reagent/mol poly(A) adenylate residues.. Alkylation at 20°C and 37°C was performed in a similar way, the incubation time being 7 days and 8 h, respectively.

Alkylation of nucleic acids inside the cell was studied using Krebs 2 ascites tumor cells maintained in CC57BR mice. The cell suspension was added to the reagent dissolved in 199 medium and incubated at 5°C with periodical stirring. The suspension contained 214 pmol reagent and 1.1×10^7 cells/ml in the experiments performed at 37°C and 50 pmol reagent and 5×10^6 cells/ml in the experiments at 5°C. The incubation time is given in table 1. The cells were centrifuged at $150 \times g$ and washed out twice by centrifugation in 0.14 M NaCl for 10 min in the cold. Nucleic acids were extracted from the cells by treatment with phenol. RNA was extracted from aqueous layer and interphase as in [14]. Total RNA was fractionated on the poly(U)–Sepharose column according to [15]. The fraction not bound to poly(U)–Sepharose is further referred to as fraction 1. The fraction of mRNA-containing poly(A) tracts was treated with RNase A and T_1 [16]. Poly(A) fraction (fraction 2) was isolated using poly(U)–Sepharose [15]. The remaining digest is further referred to as fraction 3. DNA was extracted from the above interphase with 0.5% SDS as in [17], at 75°C for 10 min (instead of 65°C and 5 min in the original procedure).

To identify the alkylated bases in RNA fractions and in poly(A) modified in vitro the samples were hydrolyzed in 1 M HCl at 100°C for 1 h. Adenine containing fraction 2 and poly(A) modified in vitro were pre-treated with 0.6 M NaOH at 100°C to convert the alkylated adenines into acid-stable derivatives. Alkylated purines in DNA were eliminated at pH 6, 60°C and incubated at pH 4 at 40°C to split the acetal bond between alkylated bases and the oligonucleotide moiety of the reagent. Alkylated cytosines were obtained by acid treatment of DNA with 1 M HCl at 100°C for 1 h. In all cases the alkylated bases as well as alkylated cytidine-3′(2′)phosphate were identified by paper chromatography in 3 systems according to R_F values. The details of the identification procedure may be seen in [18–20].

3. Results and discussion

Mixed oligonucleotides containing deoxyribose-phosphate backbone with 3′-terminal ribonucleoside residue were proposed as carriers of the reactive 2′,3′-*O*-4-(*N*-2-chloroethyl-*N*-methylamino)-benzylidene moiety for the complementary addressed alkylating reagents [21]. Here we have used a similar carrier to elucidate the possibility of the complementary addressed modification inside the cell using a non-ionisable oligonucleotide analog with ethylated internucleotide phosphates. 5′-Dianilido-phosphate of completely *p*-ethylated tetrathymidilyl (3′→5′)uridine was taken as starting oligonucleotide derivative. 4-(*N*-2-chloroethyl-*N*-methylamino)-[^{14}C]benzaldehyde was attached to this oligonucleotide as in [5,12] to produce the reagent I. This derivative was expected to be complementary to poly(A) tracts widely represented as 3′-terminal sequences in eukaryotic mRNAs.

Prior to the study of alkylation inside the cell we have investigated poly(A) alkylation in vitro. The reagent was incubated at 5°C with 0.17 mM poly(A) (concentration of pA residues) for 36 days and the extent of alkylation was determined as in section 2. The time is sufficient to convert nearly 75% of the 2-chloroethylamino groups of the reagent to the intermediate ethyleneimmonium cation. The dependence of the extent of alkylation on the reagent concentration is shown in fig.1. The efficiency of alkylation determined as the ratio of the alkylation rate to the rate of side-processes divided by the nucleotide residues concentration was found to be 2.3×10^3 M^{-1}. This value is 300-times greater than expected for the direct interaction of the reagent of this type with polynucleotides thus proving that the reaction proceeds mainly in the complex of poly(A) with the reagent I. No measurable modification was found in the similar experiment performed at 20°C and 37°C, temperatures exceeding the expected melting point of the complex.

To reveal the possibility of the complementary addressed alkylation inside cells, Krebs ascites tumor

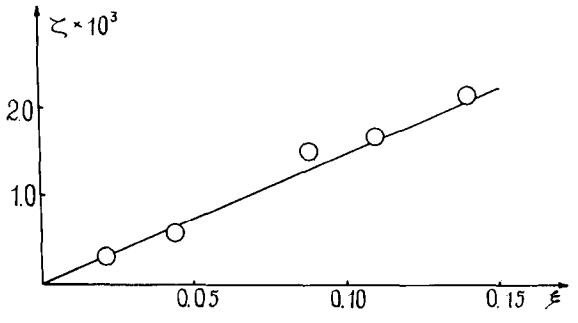


Fig.1. Dependence of the extent of alkylation of poly(A) at 5°C (mol reagent I covalently attached/mole nucleotide residues) on the ratio of initial concentrations of I and nucleotide residues of poly(A), ϵ_0.

Table 1
Degree of modification and composition of product of alkylation of nucleic acids in Krebs ascites tumor cells

Nucleic acid	T (°C)	Incubation time	Degree of modification (mol I residues mol nucleotide residues)	Composition of products of alkylation (%)		
				Guanine	Cytosine	Adenine
Fraction 1	5	17 days	4.0×10^{-5}	41	30	29
Fraction 2	5	17 days	1.5×10^{-3}	–	–	100
Fraction 3	5	17 days	5.0×10^{-5}	48	28	24
Fraction 4	5	17 days	1.1×10^{-4}	54	11	35
RNA	37	7 h	3.0×10^{-5}	82	18	–
DNA	37	7 h	1.6×10^{-4}	90	10	–

cells were treated at 5°C and at 37°C with the reagent I. The cells absorb nearly 40% of the reagent introduced in the incubation mixture. Up to 80% of the reagent absorbed by the cell were found to be covalently attached to acid-insoluble material after 17 days incubation at 5°C as well as after 7 h incubation at 37°C. Total RNA extracted from the cells treated at 5°C was fractionated by chromatography on poly(U)–Sepharose. The fraction not retained on the column is further referred as fraction 1. The absorbed fraction was treated after elution by RNase A and T_1 and repeatedly put on the poly(U)–Sepharose column to separate poly(A) tracts (fraction 2) from the remaining digest (fraction 3). The extent of modification of these fractions as well as of total DNA are given in table 1. It is seen that the extent of alkylation of poly(A) tracts is 15-times greater than that of the RNA remaining and 7-times greater than the extent of modification of DNA.

In table 1 the results of the determination of the distribution of the label among the alkylated bases are given. It is seen that 3 bases namely guanine, cytosine and adenine are modified in RNA as it was earlier shown for the complementary addressed alkylation in vitro with the derivatives of non-esterified oligonucleotides [18]. In the same time at 37°C guanine residues of RNA are predominantly modified inside the cell which is typical of the non-addressed alkylation [22].

DNA from the cells treated at 5°C was found to be alkylated mainly at guanine and adenine residues which is typical of the complementary addressed alkylation with the oligonucleotide derivatives with the reactive group attached at the 3′-end [19]. Again DNA from the cells treated at 37°C contains mainly alkylated guanines as was found for direct alkylation of DNA without intermediate complex formation [23].

3-Alkyladenine was found to be the major product of adenine alkylation in the poly(A) tracts isolated from the cells treated at 5°C as well as in poly(A) alkylated in vitro (62% and 64%, respectively). 7-Alkaladenine is converted in the course of alkaline treatment to a derivative with a split imidazole ring and 1-alkyladenine is converted to N^6-alkyladenine by the Dimroth rearrangment. Both products were found in amounts $\leqslant$20% of each in poly(A) alkylated in the cell and in vitro.

Thus it may be concluded that the reagent I is sufficiently hydrophobic to penetrate the outer cell membrane and is able to alkylate specifically complementary poly(A) sequences in the cell at a temperature lower than the melting point of the complex between the reagent and poly(A). According to the distribution of the covalently bound reagent among different bases in RNA lacking long poly(A) sequences as well as in DNA this modification seems also to proceed in the complexes at 5°C. This may be due to existence in these nucleic acids of short, single-stranded oligo(A) fragments.

References

[1] Belikova, A. M., Zarytova, V. F. and Grineva, N. I. (1967) Tetrahedron Lett. 3557–3562.
[2] Grineva, N. I. and Karpova, G. G. (1973) FEBS Lett. 32, 351–355.
[3] Grineva, N. I., Karpova, G. G., Myzina, S. D. and Chemassova, A. N. (1975) Bioorg. Khim. 1, 1707–1715.
[4] Grineva, N. I., Karpova, G. G., Kuznetsova, L. M., Venkstern, T. V. and Bayev, A. A. (1977) Nucleic Acids Res. 4, 1609–1631.

[5] Grineva, N. I. and Zarytova, V. F. (1968) Izv. Sib. Otd. Akad. SSSR ser. khim. issue 5, 125–129.
[6] Grineva, N. I. and Lomakina, T. S. (1972) Zhur. Obshch. Khim. 41, 1630–1634.
[7] Salganik, R. I., Dianov, G. L., Kurbatov, V. A., Shishkin, G. V. and Gall, A. A. (1978) Dokl. Akad. Nauk SSSR 239, 217–219.
[8] Summerton, J. and Bartlett, P. A. (1978) J. Mol. Biol. 122, 145–162.
[9] Miller, P. S., Barrett, J. C. and Ts'o, P. O. P. (1974) Biochemsitry 13, 4887–4896.
[10] Pless, R. C. and Ts'o, P. O. P. (1977) Biochemistry 16, 1239–1250.
[11] Miller, P. S., Baitermann, L. T. and Ts'o, P. O. P. (1977) Biochemistry 16, 1988–1996.
[12] Ryte, V. K., Karpova, G. G. and Grineva, N. I. (1977) Bioorg. Khim. 3, 31–38.
[13] Grineva, N. I., Zarytova, V. F. and Knorre, D. G. (1970) Zhur. Obshch. Khim. 40, 215–222.
[14] Georgiev, G. P. (1968) in: Chemistry and biochemistry of nucleic acids (Zbarshy, I. B. and Debov, C. C. eds) pp. 96–98, Medicina, Leningrad.
[15] Philipson, L., Wall, R., Glickman, G. and Darnell, J. E. (1971) Proc. Natl. Acad. Sci. USA 68, 2806–2809.
[16] Edmonds, M. and Caramela, M. G. (1969) J. Biol. Chem. 244, 1314–1324.
[17] Miller, D. (1976) Experiments in molecular genetics, pp. 309–310, Mir, Moskow.
[18] Grineva, N. I. and Karpova, G. G. (1975) Bioorg. Khim. 1, 588–597.
[19] Benimetskaya, L. Z., Karpova, G. G. and Grineva, N. I. (1978) Bioorg. Khim. 4, 1372–1381.
[20] Gimautdinova, O. I., Grineva, N. I., Karpova, G. G., Lomakina, T. S. and Shelpakova, E. L. (1978) Bioorg. Khim. 4, 917–927.
[21] Zarytova, V. F., Sokolova, N. I., Grineva, N. I., Knorre, D. G. and Shabarova, Z. A. (1968) Izv. Sib. Otd. Akad. SSSR ser. khim. issue 6, 102–105.
[22] Belikova, A. M. and Grineva, N. I. (1971) Izv. Sib. Otd. Akad. Nauk SSSR ser. khim. issue 5, 119–127.
[23] Grineva, N. I., Lomakina, T. S., Myzina, S. D. and Nogovitsina, G. K. (1974) Izv. Sib. Otd. Akad. Nauk SSSR ser. khim. issue 6, 125–136.