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Penetration of Short Fluorescence-Labeled Peptides into the Nucleus in HeLa Cells and *in vitro* Specific Interaction of the Peptides with Deoxyribooligonucleotides and DNA

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Abstract—Marked fluorescence in cytoplasm, nucleus, and nucleolus was observed in HeLa cells after incubation with each of several fluorescein isothiocyanate-labeled peptides (epithalon, Ala-Glu-Asp-Gly; pinealon, Glu-Asp-Arg; testagen, Lys-Glu-Asp-Gly). This means that short biologically active peptides are able to penetrate into an animal cell and its nucleus and, in principle they may interact with various components of cytoplasm and nucleus including DNA and RNA. It was established that various initial (intact) peptides differently affect the fluorescence of the 5,6-carboxyfluorescein-labeled deoxyribooligonucleotides and DNA–ethidium bromide complexes. The Stern–Volmer constants characterizing the degree of fluorescence quenching of various single- and double-stranded fluorescence-labeled deoxyribooligonucleotides with short peptides used were different depending on the peptide primary structures. This indicates the specific interaction between short biologically active peptides and nucleic acid structures. On binding to them, the peptides discriminate between different nucleotide sequences and recognize even their cytosine methylation status. Judging from corresponding constants of the fluorescence quenching, the epithalon, pinealon, and bronchogen (Ala-Glu-Asp-Leu) bind preferentially with deoxyribooligonucleotides containing CNG sequence (CNG sites are targets for cytosine DNA methylation in eukaryotes). Epithalon, testagen, and pinealon seem to preferentially bind with CAG- but bronchogen with CTG-containing sequences. The site-specific interactions of peptides with DNA can control epigenetically the cell genetic functions, and they seem to play an important role in regulation of gene activity even at the earliest stages of life origin and in evolution.

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Key words: short biologically active peptides, peptide–oligonucleotide (DNA) binding, DNA methylation, regulation of genetic functions

Peptides form the extensive and diverse regulatory signal system controlling physiology, growth, and development of animals and plants. In animals this is well known for relatively large peptides, most of which are hormones (neuropeptides, growth hormones, and many others). The functional role of very small peptides, in particular those consisting of 2-4 amino acid residues, was unclear for a long period and underestimated. Nevertheless, short peptides as signal molecules can trigger or inhibit various genetic processes and biochemical reactions in the cell. Under the influence of short biologically active peptides the mean lifespan of experimental animals was increased by 30-40% and growth of spontaneous, induced, and transplanted tumors was suppressed [1]. After *in vivo* introduction of peptides Glu-Trp, Lys-Glu, Ala-Glu-Asp-Gly, and Ala-Glu-Asp-Pro in mice significant changes in gene expression were observed in myocardium and brain [1]. In transgenic mice these peptides inhibit by 2-4-fold the expression of breast cancer gene *HER-2/neu*, which correlates with decrease in adenocarcinoma size [1]. In mice and rats short peptides increase transcription of *IL-2* and *c-fos* genes in lymphocytes and various structures of the hypothalamus, this

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DNA–EB, DNA–ethidium bromide complex; FAM, 5,6-carboxyfluorescein; FITC, fluorescein isothiocyanate.

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mainly stipulating immunomodulating, oncomodifying, and stress protecting properties of these peptides [1]. Geroprotecting action of short peptides is associated with chromatin activation in blood lymphocytes of senile patients [1]. The treatment of human fibroblasts with peptide Ala-Glu-Asp-Gly induced telomerase activity and extended the length of telomeres by 2.5-fold, this being accompanied with an increase in cell division number by 42.5%, thus Hayflick's limit was surmounted [1]. In aged and senile patients, the investigated short peptides increase the melatonin level, improve different physiological functions, and decrease mortality by 2-fold (for an 8-12-year period of randomized clinical observation) [1]. Some fluorescence-labeled short peptides are able to penetrate into animal cells, and they were detected in cytoplasm and accumulated around the nucleus [2], but it was still unknown whether these peptides were able to penetrate into the nucleus.

It has been assumed that tissue- or gene-specific binding of short peptides with DNA is a fundamental principle of their physiological action [1]. In fact, site-specific peptide binding with DNA can really be discussed. This is called allosteric interaction of peptides with DNA [3]. Some weighty evidences appeared showing that binding of relatively short peptides can be really site-specific, depending on primary structures of peptides and respective DNA sites (sequences) recognized [3, 4]. Binding of short peptides in the large DNA groove is accompanied by significant local changes in the DNA double helix structure [1] and gene expression. However, detailed mechanisms of such selective binding of short peptides with DNA and of resulting induction or repression of gene expression are still poorly investigated. In spite of many available data on the existence and formation of various specific short peptide–DNA complexes [1, 3-7], the site specific interaction and binding of such peptides and their possible penetration into the cell nucleus are still often called in question. Anyhow, the unique peculiarities and rules of the possible biological result producing binding of primary structures of DNA chains and short peptides are still practically unknown.

In the present work the intracellular localization of various fluorescence-labeled short biologically active peptides in HeLa cells (after cell incubation in the presence of fluorescein isothiocyanate (FITC)-labeled peptides) and the *in vitro* interaction (binding) of peptides with different synthetic single- and double-stranded deoxyribooligonucleotides and λ phage DNA–ethidium bromide complexes (DNA–EB) were investigated. Interaction of peptides with oligonucleotides and DNA–EB was judged by the influence of peptides on the fluorescence spectra of deoxyribooligonucleotides labeled with 5,6-carboxyfluorescein (FAM) and on the fluorescence spectra of DNA–EB. On the other side, the influence of DNA on the fluorescence spectra of FITC-labeled peptides was studied also.

MATERIALS AND METHODS

We have used peptides synthesized at the St. Petersburg Institute of Bioregulation and Gerontology of the Russian Academy of Medical Sciences such as epithalon (Ala-Glu-Asp-Gly), pinealon (Glu-Asp-Arg), bronchogen (Ala-Glu-Asp-Leu), testagen (Lys-Glu-Asp-Gly), cardiogen (Ala-Glu-Asp-Arg), and pancragen (Lys-Glu-Asp-Trp). The purity of the synthetic peptides was analyzed, and the peptides were additionally purified by means of chromatography in a BioLogic DuoFlow (USA) apparatus on a C-18 column in the concentration gradient (0-60%) of acetonitrile containing 1% trifluoroacetic acid. Deoxyribooligonucleotides of known primary structure and labeled with FAM were synthesized and kindly presented to us by Syntol (Russia).

Peptides labeled with FITC were obtained by addition of FITC solution (1.5 mg/ml) in 0.5 M sodium bicarbonate to peptide solution (1 mg/ml). The reaction mixture was incubated at room temperature for 30 min with rotary agitation. The fluorescence-labeled peptides obtained were analyzed and purified from free FITC by chromatography in the BioLogic DuoFlow apparatus as described earlier (Fig. 1). The fluorescence emission spectra were recorded in a Perkin Elmer LS 55 fluorescence spectrometer (USA).

DNA of λ phage was purchased from Fermentas (Lithuania); unlike unmethylated λ phage DNA (dcm$^-$,

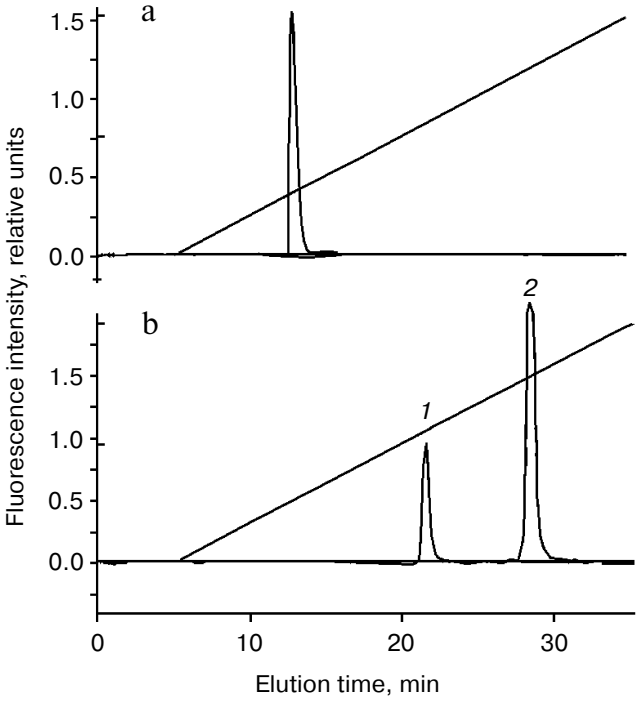

Fig. 1. Highly efficient liquid chromatography of epithalon (a) and its FITC-derivative (b) on a C-18 column in acetonitrile gradient containing 1% trifluoroacetic acid: *1*) FITC; *2*) FITC-epithalon.

dam$^-$), methylated λ phage (dcm$^+$, dam$^+$) DNA contains 5-methylcytosine residues in sequences Cm^{5}CWGG and N^6-methyladenine residues in Gm^{6}ATC sites.

To investigate possible penetration into the cell and intracellular localization of short peptides, HeLa cells were incubated for 12 h with peptides labeled with FITC ($1.2 \cdot 10^{-6}$ and $1.2 \cdot 10^{-7}$ M), washed with phosphate buffer, fixed with 1.8% formaldehyde for 15 min, incubated with 4′,6-diamidino-2-phenylindole (DAPI) (0.1 μg/ml) for 10 min, and trapped into Mowiol. The preparations were investigated and photographed in an Axiovert 200M fluorescence microscope (Zeiss, Germany) with cooled Orca II-Erg CCD-camera (Hamamatsu, Japan). Contours of nuclei were detected with DAPI treatment. The mean intranuclear fluorescence values were determined using the ImageJ program.

RESULTS AND DISCUSSION

After incubation of HeLa cells in the presence of FITC-labeled peptides, significant fluorescence of many small granules (probably endosomes) in the cytoplasm was observed. This corresponds to data that showed short fluorescence-labeled peptides can penetrate into animal cells [2]. A marked fluorescence was observed also in nuclei of HeLa cells (Figs. 2 and 3). The relative intensity of fluorescence of various peptides in nuclei of HeLa cells is different (Fig. 3). In particular, the fluorescence is markedly expressed in nuclei of cells incubated with fluorescence-labeled pinealon and epithalon and to lesser degree with testagen (Figs. 2 and 3). The differences in the fluorescence intensity of nuclei observed can be due to different degree of penetration of various fluorescence-labeled peptides into the cell and intracellular structures, different degree of peptide binding with these structures and their components in the nucleus, and different degree of quenching of fluorescence of labeled peptides used during their interaction with nuclear structures. In fact, we did not expect to observe very intense fluorescence of labeled peptides in nuclei. The nucleus does not seem to serve as a depot for such short peptides, and higher peptide concentrations are unnecessary for performance of their signal functions in the possible regulation of gene activity, particularly on binding with DNA. Unlike the relatively discrete character of fluorescence distribution

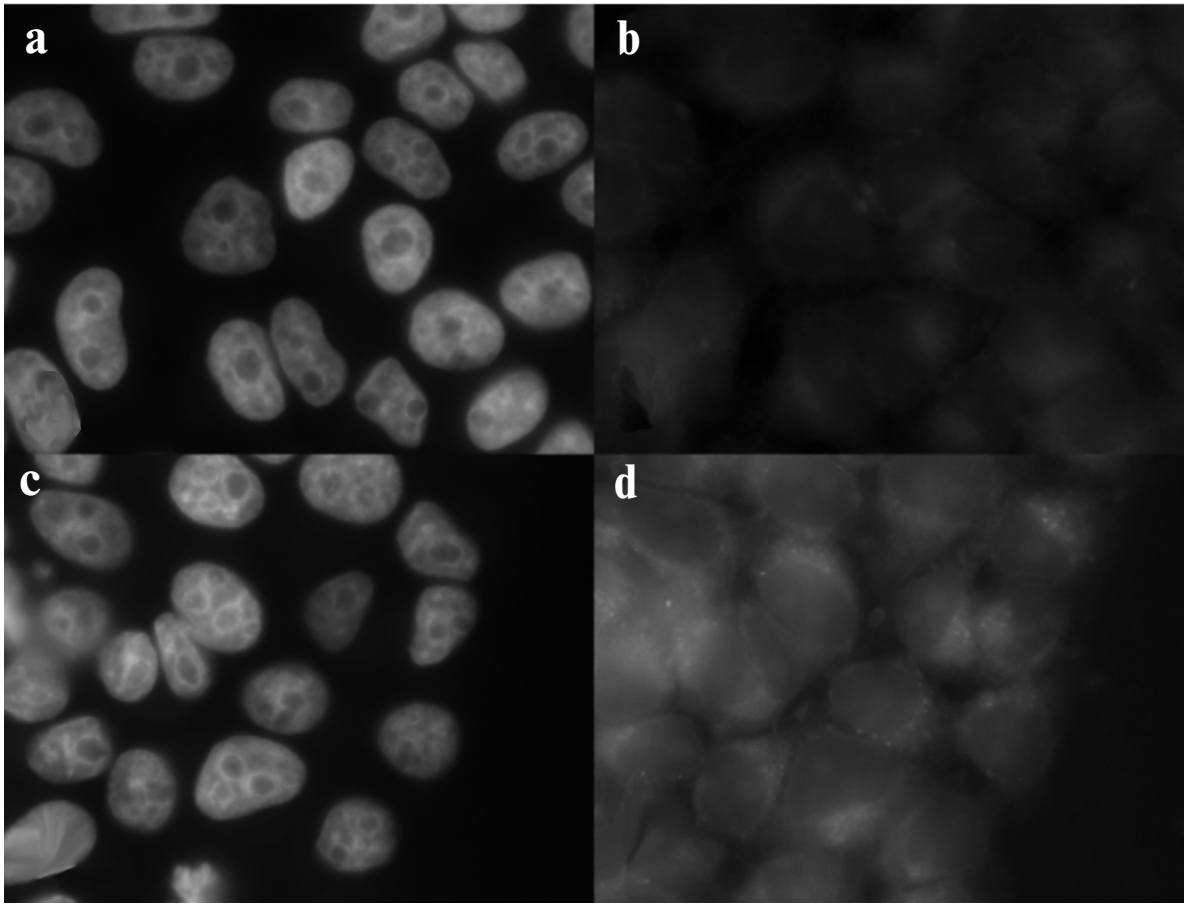

Fig. 2. Fluorescence of FITC-conjugated pinealon in nuclei of HeLa cells: a, b) control; c, d) after 6 h incubation of cells with FITC-conjugated pinealon ($1.2 \cdot 10^{-6}$ M); a, c) after cell treatment with DAPI; b, d) fluorescence of FITC-conjugated pinealon in nuclei.

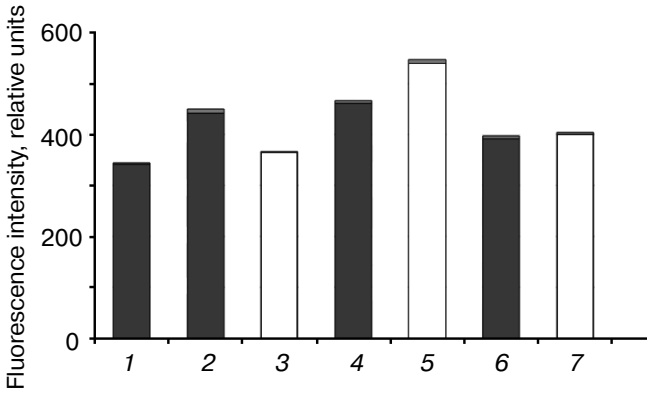

Fig. 3. Morphometric analysis of content of fluorescence-labeled peptides in nuclei of HeLa cells after 6 h cell incubation with FITC-peptides. Mean intensity of the nuclei fluorescence (relative units). *1*) Control; *2*, *3*) epithalon; *4*, *5*) pinealon; *6*, *7*) testagen. FITC-peptide concentration in incubation medium: black columns – $1.2 \cdot 10^{-6}$ M; open columns – $1.2 \cdot 10^{-7}$ M. Grey areas in the upper part of columns – standard error.

in cytoplasm, the fluorescence in nucleoplasm is relatively homogenous, but it is more expressed in the nucleolus (Fig. 2). Thus, the fluorescence-labeled peptides used can penetrate into an animal cell and its nucleus and nucleolus. In principle, this is an obligatory event for their possible interaction with nucleic acids of the nucleus and nucleolus. Detection of significant fluorescence in a nucleolus shows that short peptides potentially have the possibility to interact with both DNA and RNA (the nucleolus is the place of RNA synthesis and, in particular, ribosomal RNA).

Investigation of interaction of fluorescence-labeled deoxyribooligonucleotides with short biologically active peptides showed that peptides having different primary structures bind with one and the same deoxyribooligonucleotide differently. In Fig. 4 the fluorescence spectra of deoxyribooligonucleotide FAM-GCG GCG TGA TCA GCG GCG GCG without and after titration with epithalon (Ala-Glu-Asp-Gly) or cardiogen (Ala-Glu-Asp-Arg) are represented. It is clearly seen that epithalon, unlike cardiogen, strongly quenches fluorescence of this oligonucleotide and, therefore, interacts with it.

Static quenching of fluorescence of a labeled deoxyribooligonucleotide on formation of an oligonucleotide–peptide complex can be described by the Stern–Volmer equation [8, 9]:

$$F_o/F = 1 + K_{quench}\,[Q],$$

where F_o is initial intensity of fluorescence, F is fluorescence intensity after addition of quenching agent, [Q] is molar concentration of quencher (peptide), K_{quench} is a constant of fluorescence quenching that is determined as an angle of inclination of respective straight line on the Stern–Volmer graph.

In Fig. 5 the Stern–Volmer graphs of quenching of fluorescence of deoxyribooligonucleotides FAM-GCG GCG CAT GCG GCG GCG, FAM-GCG GCG GAT ACG GCG GCG, FAM-GCG GCG GAT CCG GCG GCG GCG and FAM-GCG GCG TGA TCA GCG GCG by epithalon are presented. These graphs have linear character that indicates the static quenching of fluorescence. Based on these graphs, the Stern–Volmer con-

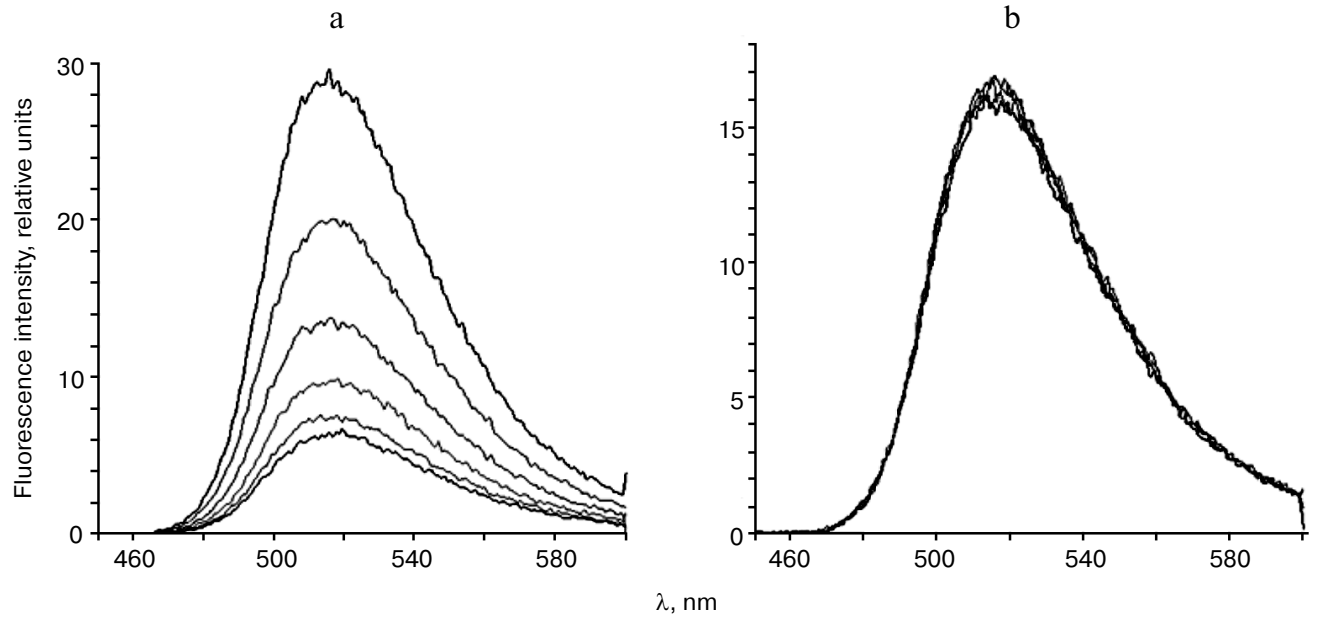

Fig. 4. Fluorescence spectra of FAM-GCG GCG TGA TCA GCG GCG GCG oligonucleotide on light (λ = 430 nm) excitation: a, b) titration of the fluorescence-labeled oligonucleotide by epithalon or cardiogen, respectively.

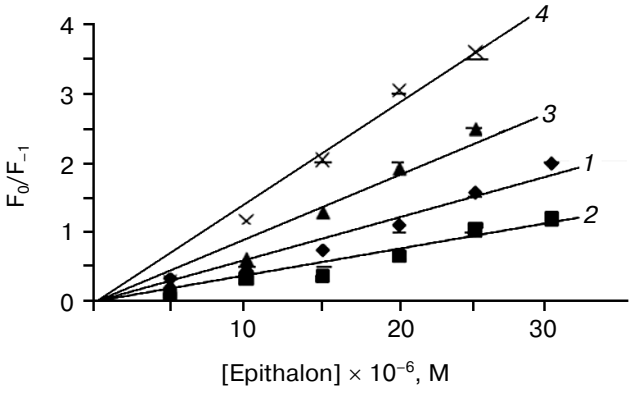

Fig. 5. Stern–Volmer graphs obtained (calculated) on titration by epithalon (Ala-Glu-Asp-Gly) of the fluorescence-labeled oligonucleotides: *1*) FAM-GCG GCG GAT GCG GCG GCG; *2*) FAM-GCG GCG GAT ACG GCG GCG; *3*) FAM-GCG GCG GAT CCG GCG GCG GCG; *4*) FAM-GCG GCG TGA TCA GCG GCG.

stants were calculated (Table 1); these constants characterize the interaction degree of all peptides investigated with different deoxyribooligonucleotides. The Stern–Volmer constants of fluorescence quenching of labeled deoxyribooligonucleotides by peptides differ significantly depending on amino acid sequences of the interacting peptides. This indicates the existence of specific interaction of various short peptides with nucleic acid structures. Cardiogen (Ala-Glu-Asp-Arg) did not quench fluorescence of any single-stranded monotonous oligonucleotides (Fig. 4b and Table 1), and it only slightly quenched fluorescence of some double-stranded oligonucleotides. Therefore, cardiogen seems not to bind with single-stranded oligonucleotides and relatively monotonous nucleotide sequences. Pinealon (Glu-Asp-Arg), which differs from cardiogen by the absence of one amino acid (Ala), induced relatively small quenching of fluorescence of all of the labeled deoxyribooligonucleotides used (Table 1). Pinealon predominantly quenches fluorescence of oligo(dT) and least of oligo(dC). Pancragen (Lys-Glu-Asp-Trp) quenches fluorescence of oligo(dA) and oligo(dT) to similar extent, and it does not influence the fluorescence of oligo(dC) and oligo(dGC). Compared with pancragen the change of one amino acid (Trp) for Ala in testagen results in an increase in the Stern–Volmer constant values by 3-4-fold. Testagen quenches also fluorescence of oligo(dC) and especially of oligo(dGC). It is interesting that bronchogen (Ala-Glu-Asp-Leu) does not bind with oligo(dGC) at all. Epithalon (Ala-Glu-Asp-Gly) that is similar to bronchogen in amino acid sequence has lower hydrophobicity index (–5.6) on the Kyte–Doolittle scale [10] compared with that of bronchogen (–1.4), and it has higher (by 1.5-2-fold) Stern–Volmer constant.

Interaction of all peptides (except for pinealon) with double-stranded oligo(dA)-oligo(dT) obtained by annealing of single-stranded oligo(dA) and oligo(dT) is characterized with lower (by 2-3-fold) Stern–Volmer constant values compared with that of single-stranded oligonucleotides. Thus, short peptides interacting with monotonous oligonucleotides prefer to bind to single-stranded structures.

In addition to monotonous oligonucleotides we have used also synthesized fluorescence-labeled deoxyribooligonucleotides containing CG and CNG sites that are known to be recognized by eukaryotic cytosine DNA-methyltransferases [11] and some specific higher plant endonucleases [12]: FAM-GCG GCG GAT GCG GCG GCG, FAM-GCG GCG GAT ACG GCG GCG, FAM-GCG GCG GAT CCG GCG GCG GCG and FAM-GCG GCG TGA TCA GCG GCG. It was established that cardiogen and pancragen do not bind with GC-enriched oligonucleotide FAM-GCG GCG CAT GCG GCG GCG as well as with oligo(dGC), but testagen and epithalon bind with it demonstrating more or less similar constants of fluorescence quenching (Table 1). On interaction with pinealon, the fluorescence quenching constant of this oligonucleotide is almost 2-fold higher compared with that observed on its binding with oligo(dGC). Bronchogen binds with FAM-GCG GCG CAT GCG GCG GCG but not with oligo(dGC). Change of one G residue for T after A in the oligonucleotide significantly decreases the Stern–Volmer constant value when this oligonucleotide interacts with all of the studied peptides, but change of G for C at the same position is accompanied with a significant increase of this constant on the interaction of the oligonucleotide with bronchogen and, particularly, with epithalon (Table 1). Pancragen was shown to slightly bind with FAM-GCG GCG GAT CCG GCG GCG GCG. Change of adenine residue for cytosine in this oligonucleotide does not affect the fluorescence quenching constant on its treatment with pinealon or testagen.

Both ionic and hydrophobic interactions play an important role during binding of short peptides with oligonucleotides. Hydrophobicity indexes of pinealon (–11.5) and testagen (–11.3) are lower than that of epithalon (–5.6) and bronchogen (–1.4), but the fluorescence quenching constants on oligonucleotide interactions with bronchogen and, particularly, with epithalon are higher than that with testagen and pinealon. Oligonucleotides with TGA and TCA sequences inside chains bind relatively well with all of the peptides including cardiogen. This again indicates the site-specific nature of short peptide binding with deoxyribooligonucleotides. In other words, depending on amino acid sequence the peptide binds with respective definite nucleotide sequence of oligonucleotides.

Epithalon (Ala-Glu-Asp-Gly) binding is sensitive to the share of cytosine or guanine in nucleotide sequences. Judging from the fluorescence quenching data, the constant of binding of epithalon with FAM-CGC CGC CAG

Table 1. Peptide interaction with FAM-deoxyribooligonucleotides (quenching of fluorescence, Stern–Volmer constants, $\times 10^4$)

FAM-deoxyribooligonucleotides	Bronchogen (AlaGlu-AspLeu)	Cardiogen (AlaGlu-AspArg)	Pancragen (LysGlu-AspTrp)	Pinealon (Glu-AspArg)	Testagen (LysGlu-AspGly)	Epithalon (AlaGlu-AspGly)
FAM-oligo(dA)	3.8	n.d.	1.0	1.2	4.1	6.3
FAM-oligo(dT)	2.9	–"–	1.6	1.8	3.6	4.0
FAM-oligo(dA) + oligo(dT)	1.3	–"–	n.d.	1.1	1.7	3.2
FAM-oligo(dC)	1.0	–"–	–"–	0.5	0.9	1.1
FAM-oligo(dGC)	n.d.	–"–	–"–	1.5	5.5	5.9
FAM-GCG GCG GATGCG GCG GCG	2.7	–"–	–"–	3.1	5.6	5.4
FAM-GCG GCG GATACG GCG GCG	1.9	–"–	–"–	0.8	3.8	3.1
FAM-GCG GCG GATCCG GCG GCG	3.8	–"–	0.8	0.8	4.4	9.7
FAM-GCG GCG TGATCA GCG GCG	5.2	0.34	1.2	4.7	10.0	14.0
FAM-CGC CGC CAG GCG CCG CCG CGC	3.9	n.d.	1.7	3.2	5.8	13.0
FAM-CGC CGC m^5CAG GCG CCG-CCG CGC	4.7	–	–	–	–	21.0
FAM-GCG CGG CGG CGC CTG GCG GCG	3.1	n.d.	0.6	0.7	2.7	4.8
FAM-GCG CGG CGG CGC m^5CTG GCG GCG	5.3	–"–	n.d.	1.7	4.2	13.0
FAM-CGC CGC CAG GCG CCG CCG CGC + FAM-GCG CGG CGG CGC CTG GCG GCG	5.4	0.6	2.0	3.4	5.5	8.9
FAM-CGC CGC CAGGCG CCG CCG CGC + FAM-CGC CGC m^5CAG GCG CCG CCG CGC	5.7	n.d.	1.5	2.4	6.1	5.7

Note: n.d., not detected; –, not determined.

GCG CCG CCG CGC (12 C residues) is almost 2-fold higher than that with FAM-GCG CGG CGG CGC CTG CGC CGC (10 C residues) (Table 1), thus the peptide predominantly binds with the oligonucleotide that has more cytosine residues. Introduction of 5-methylcytosine residue into nucleotide sequence independent of C or G content in it increases the binding of oligonucleotides with epithalon (Table 1). Thus, the binding of peptide Ala-Glu-Asp-Gly is sensitive to the cytosine methylation status of oligonucleotides. Annealing of complementary oligonucleotides both containing and not containing 5-methycytosine resulted in decrease in the Stern–Volmer constant values compared with that of the initial oligonucleotides. Epithalon (Ala-Glu-Asp-Gly) prefers to bind with single-stranded oligonucleotide containing methylated cytosine. The hydrophobicity index of testagen (Lys-Glu-Asp-Gly), in which compared with epithalon an Ala residue is substituted with a polar Lys residue, is about 2-fold less than that of epithalon. Testagen binds with oligonucleotides containing 5-methylcytosine more weakly than with the same but unmethylated oligonucleotides. Annealing of complementary oligonucleotides does not decrease their binding with testagen, unlike the binding with epithalon. In this particular case on the use of annealed oligonucleotides containing 5-methylcytosine a small increase in fluorescence quenching constants was observed. Bronchogen (Ala-Glu-Asp-Leu) behaves similarly in spite of the fact that it has the highest hydrophobicity index (–1.4). Pancragen (Lys-Glu-Asp-Trp) and pinealon (Glu-Asp-Arg) interact more or less similarly with single- and double-stranded structures relatively independently of the presence of 5-methylcytosine residues in the oligonucleotides (Table 1).

We have investigated the effect of short peptides on fluorescence of DNA–ethidium bromide (DNA–EB) complexes. In this study the unmethylated (dam^-, dcm^-) and methylated (dam^+, dcm^+) λ phage DNAs were used. Ethidium bromide itself has very low fluorescence on excitation by light of wavelength $\lambda = 540$ nm. In the presence of DNA the fluorescence strongly increased (Fig. 6) due to EB intercalation between base pairs in DNA dou-

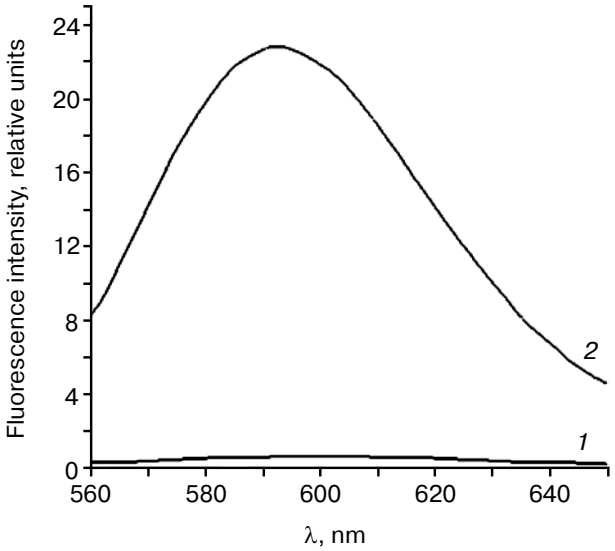

Fig. 6. Fluorescence spectra with light (λ = 540 nm) excitation of: *1*) ethidium bromide (2.2 μM); *2*) λ phage DNA (5.5 μM)–ethidium bromide (2.2 μM) complex.

ble helix. Fluorescence of DNA–EB complex can be markedly quenched by various ligands, which allows us to investigate the interaction of DNA–EB complexes with different relatively small compounds [7]. From our point of view for production of DNA–EB complexes we have chosen more or less optimal (based on fluorescence intensity) ratio of components in a mixture: DNA (5.5 μM) and EB (2.2 μM).

After addition of epithalon (Ala-Glu-Asp-Gly) to unmethylated phage DNA–EB complex, the fluorescence of this complex was quenched immediately and the maximum of the emission spectrum (λ_{max}) was shifted by more than 4 nm (from 591.8 to 596.1 nm) (Fig. 7a). It seems that this is a result of intercalation of epithalon into two-stranded DNA structure and its competition with EB for binding with DNA. Subsequent titration of the DNA–EB complex with epithalon is accompanied by only insignificant quenching of fluorescence (up to peptide concentration $2.5 \cdot 10^{-5}$ M). And the Stern–Volmer graph was represented by broken lines. Therefore, the fluorescence quenching constants were calculated only for the area where the proper quenching of fluorescence proceeded (Table 2). Estimated in this way, the constant of fluorescence quenching of λ phage DNA (dam^-, dcm^-)–EB complex (Table 2) induced by epithalon is more than 4-fold less than that on epithalon interaction with the low molecular mass deoxyribooligonucleotides including annealed ones (Table 1).

Character of the fluorescence quenching of complexes methylated phage DNA–EB and unmethylated phage DNA–EB induced by epithalon is different (Fig. 7). At concentrations lower than $1.5 \cdot 10^{-5}$ M, epithalon did not affect fluorescence of unmethylated DNA–EB complex. It seems that epithalon at relatively low concentrations is unable to compete with ethidium bromide for binding with DNA. But on subsequent addition of epithalon a sharp fluorescence quenching with 5 nm bathochromic shift of the fluorescence maximum occurred. On titration of methylated DNA–EB complex, strong fluorescence

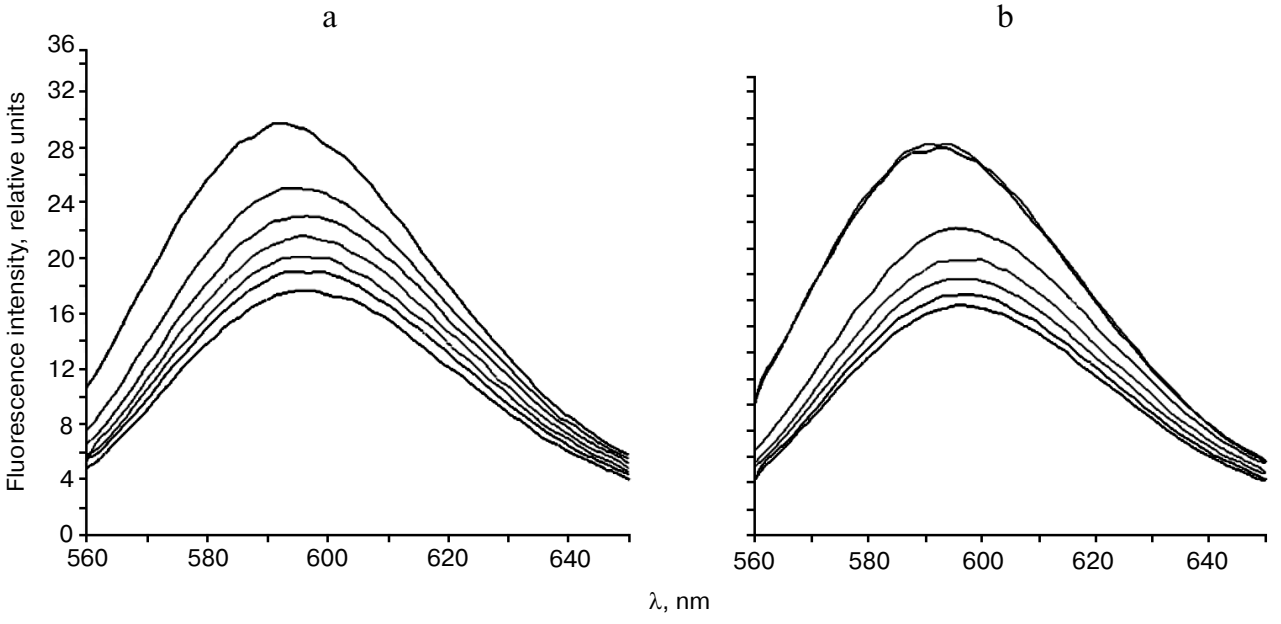

Fig. 7. Fluorescence spectra on light (λ = 540 nm) excitation of complexes of EB–unmethylated λ phage DNA (a) and EB–methylated λ phage DNA (b) without and on titration with epithalon.

Table 2. Peptide interaction with fluorescent ethidium bromide (EB)–λ phage DNA complexes (Stern–Volmer constants, $\times 10^4$)

Peptide	EB–λ phage DNA (dam^-, dcm^-)	EB–λ phage DNA (dam^+, dcm^+)
Bronchogen	0.98	0.95
Cardiogen	3.0	2.8
Pancragen	0.51	0.79
Pinealon	0.67	1.1
Testagen	0.76	0.75
Epithalon	2.1	1.9

quenching and 7 nm bathochromic shift of the fluorescence maximum were observed immediately even at low epithalon concentrations. Saturation with epithalon of methylated DNA–EB complex proceeds at lower peptide concentrations than on titration with epithalon of complex unmethylated DNA–EB. At least, two types (sites) of epithalon binding with DNA–EB complexes were observed on construction of Stern–Volmer graphs. The Stern–Volmer constants for straight plots of graphs of epithalon interaction with unmethylated and methylated DNAs are practically the same (Table 2). It seems that the epithalon binding with one of two DNA regions does not depend on DNA methylation status, but binding with another DNA region does.

All other peptides studied except for cardiogen have similar character of fluorescence quenching on titration of DNA–EB complex. The fluorescence quenching of methylated DNA–EB and unmethylated DNA–EB complexes by cardiogen (Ala-Glu-Asp-Arg) is demonstrated well with Stern–Volmer graphs, and they have straight linear character. The fluorescence quenching constants of these complexes on titration with cardiogen are 1.5-6.0-fold higher than that with the other peptides (Table 2). Besides, bathochromic shift of the maximum of fluorescence induced with cardiogen is about 2 nm more than that observed under the influence of epithalon (Fig. 8). These cardinal changes in the emission spectra of DNA–EB complexes under the influence of cardiogen are especially surprising because addition of this peptide to single-stranded synthetic oligonucleotides does not induce any changes in their fluorescence spectra. These phenomena might be explained by the probability that cardiogen is able to bind only to double-stranded DNA structures.

Along with investigation of effects of the intact peptides on fluorescence of DNA–EB complexes, we have studied also how these particular complexes can influence the fluorescence of proper fluorescence-labeled peptides. To some extent it was a sort of reverse task. For this work the FITC derivatives of peptides were synthesized and purified by liquid chromatography on a C-18 column in a concentration gradient of acetonitrile similarly as previously described. FITC-labeled peptides are more hydrophobic than the initial peptides and free FITC; therefore, they were eluted from the column at higher acetonitrile concentration (Fig. 1). Peptides with diamino acids such as cardiogen, pancragen, pinealon, and testagen can bind FITC with all their free amino groups and not only with the N-terminal amino group. We have used only the respective mono-FITC derivatives of the peptides.

Titration of fluorescent derivative of epithalon with unmethylated λ phage DNA does not decrease the peptide fluorescence (Fig. 9a). But methylated phage DNA slightly quenches the fluorescence of labeled epithalon. Unlike the results on titration of DNA–EB complex with epithalon, the titration of FITC-labeled epithalon with DNA was not accompanied with bathochromic shift of the maxima of the emission spectra. Because unmethylated DNA is more polar than methylated DNA, this seems to significantly influence the interaction of DNA with the hydrophobic derivative of epithalon. It might be that methylated bases in DNA are located in (or near) regions of DNA binding with epithalon. It seems that these DNA regions are inaccessible for binding with hydrophobic FITC-labeled epithalon. FITC-epithalon interacts preferentially with more hydrophobic methylated DNA compared with more polar unmethylated DNA. The Stern–Volmer constants on interaction of FITC-labeled epithalon with DNA are 5-10-fold higher than that on

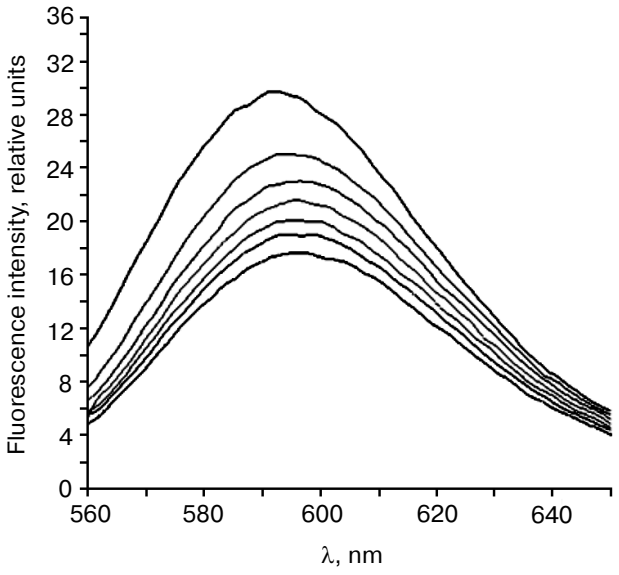

Fig. 8. Fluorescence spectra on light (λ = 540 nm) excitation of EB–unmethylated λ phage DNA complex without and on titration with cardiogen.

Table 3. Effect of unmethylated and methylated λ phage DNAs on fluorescence of FITC-peptides (Stern–Volmer constants, $\times 10^4$)

FITC-peptides	λ phage DNA (dam^-, dcm^-)	λ phage DNA (dam^+, dcm^+)
FITC-bronchogen	1.9	1.6
FITC-cardiogen	3.4	2.3
FITC-pancragen	12.7	9.4
FITC-pinealon	3.1	2.2
FITC-testagen	4.5	4.8
FITC-epithalon	0.9	2.2

titration of DNA–EB complex with the initial unlabeled epithalon (Tables 2 and 3).

As shown in Table 3, the Stern–Volmer constants on binding of all of the peptides (except for epithalon) with DNA are higher than that on binding with relatively short oligonucleotides (Table 1). Binding of more polar peptides such as pancragen (–11.8), pinealon (–11.5), and testagen (–11.3) is characterized by higher Stern–Volmer constants (Table 3). Thus, they bind to DNA more strongly than the less polar peptides. The most hydrophilic peptide pancragen is most efficient on binding with DNA (Table 3). It is probable that more polar peptides interact only with DNA surface but do not intercalate into its double-helical structure. These peptides slightly prefer to bind with more polar unmethylated DNA. Constants of the fluorescence quenching of FITC-labeled cardiogen on its titration with DNA are not significantly different from that obtained on titration of DNA–EB complexes with intact cardiogen. It seems that cardiogen is able to intercalate into DNA double helix.

Thus, short biologically active peptides interact specifically with single- and double-stranded deoxyribooligonucleotides and λ phage DNA. Some of these peptides (for example, epithalon) prefer to bind with single-stranded oligonucleotides. Interaction of peptides with nucleic acid substrates depends on nucleotide sequence. In other words, there are specific sites with particular nucleotide sequence in oligonucleotide and respective amino acid sequence in peptides for mutual biopolymer binding. Some peptides (pancragen) preferentially interact with double-stranded DNA, probably by formation of bonds on the surface of the DNA molecule.

In spite of detection of specific binding of short peptides with deoxyribooligonucleotides and DNA dependent on their primary structures, unfortunately we cannot yet name the exact address of binding of any particular peptide in terms of DNA nucleotide sequence. There is so-called circuit interaction of peptides with both DNA strands, and it may be partially circuit and non-circuit binding of short peptides with DNA (peptides $(HPRK)_3NH_2$ and $(SPRK)_3NH_2$ bind with GC-enriched DNA sites without interaction between both DNA chains) [3]. There are some data on preferential binding of arginine-containing peptides with DNA enriched with AT base pairs [6].

On investigation of peptide biding with monotonous oligonucleotides, we have observed that pancragen (Lys-Glu-Asp-Trp) containing lysine was binding only with

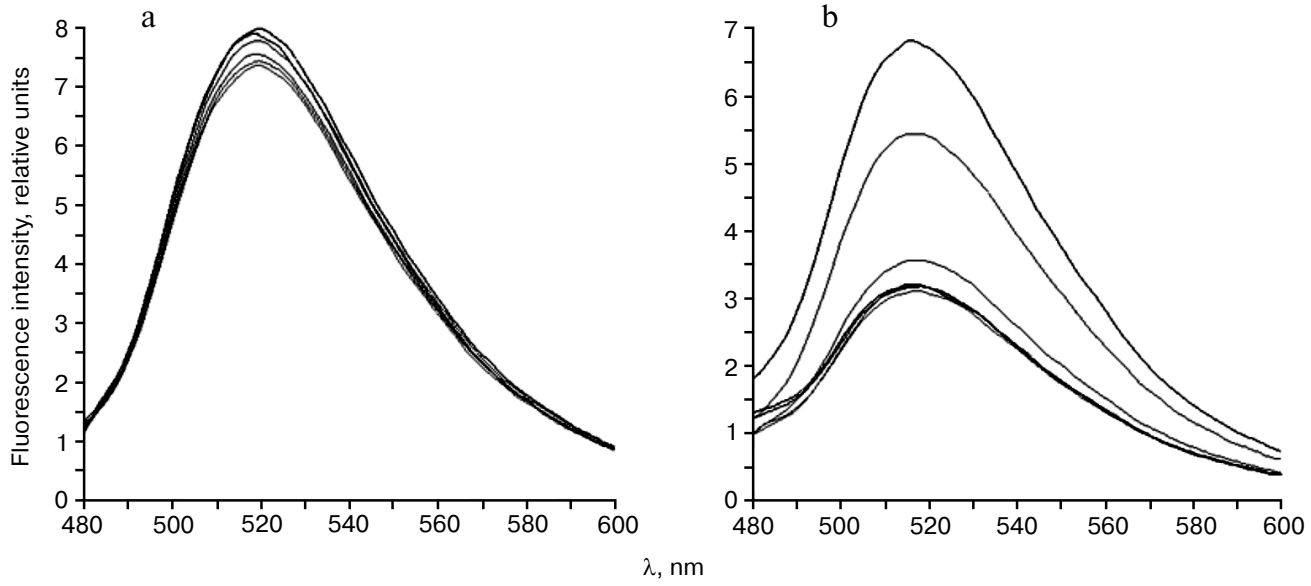

Fig. 9. Fluorescence spectra on light (λ = 430 nm) excitation of FITC-labeled epithalon (a) and FITC-labeled pancragen (b) without and on titration by unmethylated λ phage DNA.

oligo(dA) and oligo(dT) (Table 1). However, lysine-containing testagen (Lys-Glu-Asp-Gly) was binding with all monotonous oligonucleotides but preferentially with oligo(dGC). Pinealon (Glu-Asp-Arg) containing arginine also prefers to bind with oligo(dGC). Though cardiogen (Ala-Glu-Asp-Arg) contains arginine, it does not bind with single-stranded oligonucleotides. Epithalon (Ala-Glu-Asp-Gly) and bronchogen (Ala-Glu-Asp-Leu) that do not have diamino-monocarboxylic amino acids prefer to bind with oligonucleotides containing 5-methylcytosine. Epithalon, testagen, pinealon, and bronchogen bind preferentially with sequences containing CNG sites, the first three peptides preferring to bind with structures containing CAG and bronchogen with CTG sequences. It is important to note that CNG sequences are target sites for cytosine DNA methylation in plants and animals [11].

The existence of specific peptide binding with single-stranded oligonucleotides may have special significance. Single-stranded stretches are always present in DNA. In particular, they appear on DNA replication, repair, and recombination. Interaction of short peptides with such DNA stretches can specifically control the mentioned genetic processes. Besides, intercalation of short peptides (epithalon) into DNA is accompanied by local unwinding of DNA chains [1], which leads to origin of single-stranded targets for peptide binding with DNA. This may have a particular sense on possible conjugative action of different peptides in the cell when one peptide serves as an inducer (modulator) of origin of DNA single-stranded structure, but another may be a proper regulator of the biological effect.

Thus, short biologically active peptides are able to penetrate into a nucleus and to selectively bind with particular oligonucleotides and DNA nucleotide sequences *in vitro*. Besides, some of these peptides can even discriminate between nucleotide sequences with different cytosine methylation status.

We have discovered tissue, subcellular, and age specificity of DNA methylation [13] and were first to show that the DNA methylation pattern in cancerous cells is different than in normal cells [14]. Taking these data into consideration, we postulate that one and the same short biologically active peptide can bind with DNA differently depending on the DNA methylation character. Therefore, it may influence genetic processes including transcription and others in different fashion in different tissues (cells), nucleus, and mitochondria, in young and aged cells, and in normal and malignant cells. Almost all of these postulates are already proved experimentally [1].

From our point of view the site-specific peptide binding may or even should modulate the action of many proteins operating with DNA (RNA- and DNA-polymerases, DNA-methyltransferases, endonucleases, DNA repair enzymes, many regulatory protein factors, and others) competing with peptides for the same binding sites in DNA. In particular, we recently established that short peptides really modulate the action of eukaryotic CNG-site-specific endonucleases [4]. Some hexapeptides are strongly selective structural ligands for protein-free Holliday junctions, and they block recombination [15]. We suggested one of the most probable mechanisms of gene activation with short peptides [4]: selective peptide binding with CNG or CG promoter sites may protect them from the action of respective DNA-methyltransferases, and as a result the promoter will be unmethylated, which is crucial for activation of most genes.

Specific (allosteric) peptide–DNA interactions can epigenetically control genetic functions of cell, and they most probably played a very important role even at the earliest stages of life origin and in subsequent evolution.

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