Covalent DNA–protein complexes in murine cells

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DNA obtained under conditions that eliminate non-covalently bound proteins still contains small amounts of a firmly bound protein component. The nature of protein(s) (peptide(s)) which are intrinsically bound in DNA and the structural aspects of the binding of proteins in DNA are the topics of current investigation. Radiolabelled with ^{125}Iodine DNA from Ehrlich ascites tumor (EAT) and Friend's erythroleukemia (MEL) cells were examined for covalent bonds between hydroxy amino acid residues in peptides and nucleotide phosphate groups. The oligonucleotidyl-peptide fraction released from DNA after combined nuclease and protease treatment were examined for their chemical and enzymatic stability. The chemical and enzymatic stability of the linking groups and our experience with model compounds allow the conclusion that O^4-phosphotyrosine is an amino acid derivative joining the peptides covalently to DNA.

Key words: DNA–protein interaction, stable DNA–protein complexes

Abbreviations: MEL – Friend's erythroleukemia cells; PBS – phosphate buffered saline; EAT – Ehrlich ascite cells; PMSF – phenylmethyl sulfonyl fluoride; BSA – bovine serum albumin; TBD – eukaryotic non-histone proteins tightly bound to DNA

INTRODUCTION

The structural aspects of chromatin and the architecture of the nucleus are important factors in the control of gene expression [1]. During the past 20 years many investigators have shown that there are 2–5% of nucleus proteins which co-purified with DNA, although harsh deproteinisation procedures such as high salt, SDS, urea, phenol are involved [2–4]. Most of these proteins are tightly but not covalently bound to DNA [3]. We suppose that some of them can be covalently attached to DNA. Covalently DNA-bound proteins have been observed in different prokaryotic, plasmid and viral systems [5–7]. Several groups have demonstrated that the homologous covalent complexes exist in higher eukaryotes [5, 8, 9]. Up to date, all examined covalent complexes has been phosphoesters of nucleic acids and proteins. To such group belong enzymes that cleave the internucleotide bond (Topo I [10], ∅X174 protein A [11], DNA-Cre recombinase [12, 13], etc.). They belong to the polynucleotide transferase class of enzymes which form so-called relaxed complexes with nucleic acids. The covalent complexes are active for polynucleotide transfer to an acceptor molecule. No energy-rich compounds are necessary to form the internucleotide bond and to transfer the polynucleotide to an acceptor, because the interpolymeric complex itself has free energy enough to cause such reactions. The free energy of phosphodiester formed by uridylic acid and tyrosine is 10 kcal/mol [6], the amount sufficient for internucleotide bond restoration.

It is accepted that peptides are intrinsically bound in the DNA structure; it would be reasonable to assume that they are of functional significance [5, 6, 9]. The structural aspects of the covalent binding of proteins in DNA and the nature of this functional significance are the topics of current investigation. This study aims to further characterize tightly DNA-bound proteins from eukaryotic cells.

MATERIALS AND METHODS

Materials

Na^{125}J (17.4 Ci/mg) was from Amersham; fetal calf serum was supplied by Institute of Biochemistry (Lithuania). Other chemicals were of possibly highest quality.

Cell Culture

Friend erythroleukemia cells, clone F4N were grown in RPMI-1640 medium (Sigma) supplemented with 10% (v/v) heat inactivated fetal calf serum. Cells were maintained in logarithmic grown phase (1^x10^5–2^x10^6 cells /ml) with appropriate dilutions every 3–4

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days. Cell viability evaluated by a Trypan blue dye exclusion test was never lower than 95%.

Ehrlich ascites tumor (EAT) cells were propagated in mice and used 6–7 days after transplantation.

Cell lysis and salting-out procedure [14]

The cells were washed twice with ice-cold PBS and collected by centrifugation 5 min at 800 g, 4 °C, then suspended (1:6, v/v) in a suspension buffer (10 mM Tris–HCl, pH 8.2, 400 mM NaCl, 2 mM EDTA). Proteinase K (Sigma) dissolved in 1% SDS was added to the final concentration 50 μg/ml. Cell lysis was induced by additing 0.2 vol 10% SDS per 3 vol of cell suspension. The lysis mixture was sheared five times by pressing it through a 1.5 mm gauge needle and incubated for 18 h at 37 °C. Then the lysates were diluted (1:3, v/v) with a suspension buffer containing 0.63% SDS, mixed with saturated (6M) NaCl solution (3:1, v/v) and chilled 1 h before centrifugation (6000 g, 4 °C). Such crude DNA preparations were precipitated with ethanol (1:2, v/v) and suspended in 1 vol TE0.1 buffer (0.1 mM EDTA, 10 mM Tris–HCl, pH 8.0). Then 5 μg/ml DNase I-free pancreatic ribonuclease (ribonuclease A; Sigma) was added and the suspension was kept 2 h at room temperature. Such RNase-treated DNA crude preparations were kept at 4 °C or used immediately.

Isolation of nuclei and nuclear matrix

The nuclei were isolated according to the procedure of [15]. Briefly, cells were collected by centrifugation (800 g, 5 min 4 °C) and twice washed in ice-cold PBS. Cells (1^x10^7 cells /ml) were suspended in an ice-cold STM buffer (0.25 M sucrose, 10 mM Tris pH 7.4, 5 mM $MgCl_2$) containing 0.5% Nonidet P-40; the lysate was rapidly six times sheared through a 19-gauge needle and left on ice 10 min. The the latter concentration of Nonidet P-40 has been reported to dissolve outer nuclear membrane without further disruption of the nuclei. Then nuclei were sedimented (1000 g, 5 min 4 °C); the supernatant (cytosol fraction) was removed. This crude nuclear pellet was resuspended in STM buffer and further purified through 4 ml of 2.0 M sucrose cushion in TM buffer (10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$) (100,000 g, 2 h 4 °C). Then the nuclei were washed once in STM buffer and resedimented by centrifugation (5000 g, 10 min 4 °C) and lysed by resuspension in TM buffer (10 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$). Upon centrifugation (5000 g, 10 min 4 °C) a supernatant (nucleoplasm) and a particulate fraction were obtained. The latter fraction was resuspended in 500 μl of hypotonic TM buffer plus 30 U/mg DNA DNAse I and 30 U/ mg DNA RNase A. After incubation (4 °C, 1 h) 500 μl of 4M NaCl was added and resedimented. The latter fraction was termed the chromatin/matrix fraction. All buffers contained 1 mM PMSF freshly prepared from 0.1 M stock solution in anhydrous n-propyl-alcohol, 2 μM/ ml leupeptin and 2 μM/ml aprotinin.

Radiolabelling *in vitro* of residual peptides in DNA preparations with ^{125}Iodine [16, 17]

Radioiodination was proceeded as a two-step process: acylation of NH_2 groups of peptide(s) by propionil acid –N-hydrohysuccinimide ester followed by iodination of peptide(s) with $Na[^{125}I]$ / chloramine T.

Portions (5 mg) of salted-out DNA or a portion of MEL matrix fraction (corresponding to 3 x 10^7 nuclear equivalents) were precipitated with 2 vol ethanol. The DNA pellet was collected, dried and dissolved in 500 μl of H_2O. 75 μl of 10 X BES (2.5 M Na_3BO_3, pH 8.3; 50 mM EDTA; 0.5% SDS) and 150 μl of freshly prepared propionil acid –N-hydrohysuccinimide ester in isopropanol (5 mg/ml) were added. The reaction mixture was incubated for 1 h at 4 °C, then an extra portion (175 μl) of ester was added. After additional incubation (1.5 h, 4 °C) the reaction mixture was three times diluted with water and three phenol extractions (additional deproteinization steps) were achieved.

Portions of acylated and additionally deproteinized DNA from EAT (5 mg) and a portion of MEL (corresponding to 3 x 10^7 nuclear equivalents) were suspended in 200 μl of water and mixed with 6 μl (0.5 mCi) of $Na^{125}I$ (17.4 Ci per mg, NEN) and 50 μl chloramin T solution (5 mg/ml in 0.25 M Na_2HPO_4, pH 7.5). After 3 min at room temperature iodine was reduced by addition of 40 μl of sodium metabisulfate (5 mg/ml). After 5 min at 4 °C DNA was diluted with water until 1 ml and collected together with associated radiolabelled proteins by repeated (5X) precipitations with ethanol.

Isolation of nucleotide(s)-peptide(s) fractions

The pellets of radiolabelled DNA–protein fractions were digested with proteinase K (6 mg/ml) (16 h, 37 °C, 0.25% SDS, 20 mM EDTA, 150 mM $NaHCO_3$, pH 8.0). Denatured DNA was precipitated from alkaline (0.5 M NaOH) solutions, native DNA was suspended between ethanol precipitations in Tris–EDTA buffer. The residual DNA–peptide fraction was finally precipitated and the pellets were dissolved in S1 nuclease buffer (46 mM Na acetate, 10 mM Mg sulfate, pH 5.0) and digested (16 h, 37 °C with 100 mg per ml of DNaseI and 50,000 units per ml of S1 nuclease (Fermentas).

Chemical and enzymatic treatment of nucleotide(s)-peptide(s) fractions

The pellet of radiolabelled DNA–protein fractions were mixed with NaOH or HCl to obtain selected concentrations of chemicals, after treatment (usually 2 h, 56 °C) neutralized and adjusted to the optimal buffer conditions for digestion with enzymes: (1) alkaline phosphatase from calf intestine (159 U/ml, Fermentas), (2) snake venom phosphodiesterase from crotalus adamanteus venom (oligonucleate-5'-nucleotidohydrolase), EC 3.1.15.1, PDE I, 36.2 U/mg, (Sigma), (3) calf spleen phosphodiesterase (oligonucleate-3'-nucleotidohydrolase), EC 3.1.16.1, PDE II, (2 U/mg, Boehringer Manheim), (4) with proteinase K (6 mg/ml) (16 h, 37 °C, 10 mM Tris HCl, pH 7.5, 0.25% SDS, 20 mM EDTA).

Since PDE I and PDE II needed OH ends, nucleotidylpeptides were dephosphorylated with alkaline phosphatase prior to digestion with PDE I (0.01 U of PDE I per 20 µl was incubated overnight at 37 °C in buffer containing 60 mM $MgCl_2$, 30 mM Tris–HCl, pH 8.1) and PDE II (0.2 U per 20 µl of PDE II were incubated overnight at 37 °C in 75 mM ammonium acetate, pH 5.7). Following chromatography in a phenol/citrate solvent system the chromatography plates were dried and exposed to Chemapol X-ray films.

Radiochromatography

Free peptides released from DNA were detected by means of chromatography on cellulose TLC plates (Merck) using citrate-buffered phenol as solvent (phenol saturated with a solution containing 0.22 M sodium citrate and 0.27 M KH_2PO_4, pH 6.3). The chromatograms were exposed to Chemapol X-ray films.

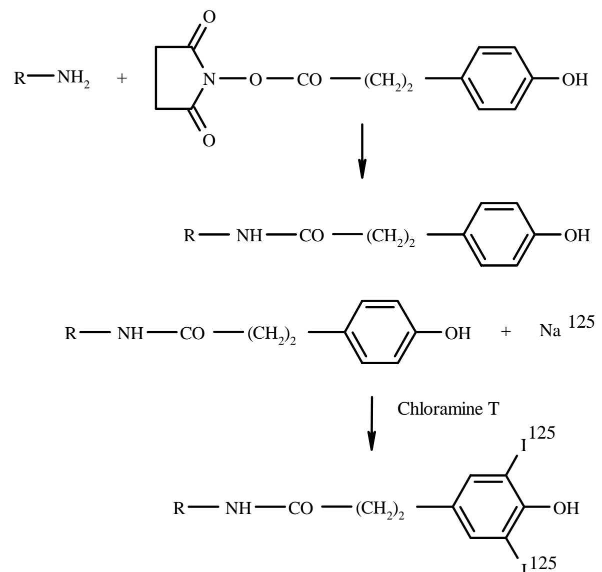
SDS-PAGE

SDS-polyacrylamide gel electrophoresis was performed using 1 mm thick gels with 10% separating gels as described by Laemmli [18]. The gels were stained with Coomassie blue R-250. All electrophoresis reagents were from Merck.

RESULTS AND DISCUSSION

Tightly DNA-bound proteins have been described by many authors [2, 6]. Although the criteria for the stability of their binding to DNA have been very different, small protein fractions always resist extractions able to destroy non-covalent associations. In this letter we report our results on covalently bound DNA–protein complexes isolated from two types of tightly bound murine chromatin: from Ehrlich ascite (EAT) and Friend's erythroleukemia (MEL) cells. In both cases DNA was isolated under conditions dissociating all peptide material, except distinct tightly bound polypeptides.

Natural covalent protein–DNA complexes are extremely difficult to isolate. They are available only in picomolar quantities. Thus radioactive labeling of the complex is necessary. As is known from literature the highest incorporation of radioactivity was obtained after selective acylation of the NH_2 groups of protein (peptide) moiety in the nucleoprotein by unlabeled β-(4-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester and subsequent iodination of 4-hydrohyphenyl, tyrosine and histidine residues by the $Na^{125}J$/Chloramine T procedure [16, 17] (Scheme I):



Scheme 1. Radioiodination scheme of DNA–protein complexes. R – aliphatic side chains of amino acids

Since the radiolabelled material remained associated with DNA during repeated treatments with hot phenol and repeated precipitations with ethanol, this showed that it behave like DNA itself. Therefore radioiodinated material of non-deoxyribonucleotide nature was chemically bound to DNA (the ratio of absorbtion at 260/280 nm was ≈1.9). However, an important point is whether these co-purifying with DNA proteins are not topoisomerases, which are known to be covalently trapped to DNA when denatured. Since it has been shown that high-salt treatment of chromatin dissociates topoisomerase II from DNA [10, 19], in isolation procedures we used a high salt treatment of chromatin to remove these enzymes from DNA.

The most important question arising from these experiments is the nature of the protein(s) covalently

linked to DNA. Attempts to fractionate them in SDS-polyacrylamide gels, even after an extensive digestion of DNA, until now have been unsuccessful [2, 3, 5]. The proteins remained at the start of the 3% stacking gel even after sequential digestions with DNAse I and phosphodiesterases. When radioiodinated EAT DNA , extensively digested with DNase I (benzonase, S1 nuclease) was subjected to 10% PAGE, the 45 kDa peptide bond was clearly seen. However, 70% of radiolabel still remains on the top of 3% stacking gel. Since after additional nuclease digestion (benzonaze, S1 nuclease) the quantity of 45 kDa peptide remains almost the same, we have no explanation as to the nature of radiolabelled material on 3% stacking gel top and 45 kDa peptide. Radiolabelled material was eluted from PAGE (3% start material and 45 kDa peptide). These materials were digested with proteinase K and the peptide(s) pattern was examined. It was confirmed that in both cases almost identical hydrolysis products (results not shown) were obtained.

A radiolabelled complex from MEL cells after extensive digestion of nucleases was obtained only on the top of 3% stacking gel.

Parallel samples of radioiodinated DNA from MEL and EAT cells isolated from phenol extractions were digested either with DNAse I or with protease K. Isolation of the core nucleotide-peptide from the initial complex was achieved by two ways: first digesting the nucleic acid component of complex and then hydrolyzing the protein component (Scheme 2, A); the opposite order of hydrolysis (Scheme II, B). Further treatment with a second protease (pronase E) of nucleotidylpeptide released no longer radiolabelled material with ^{125}J label (results not shown). We don't observed any differences between hydrolytic properties of these two sets of nucleotidylpeptides. The amount of radiolabelled material remaining acid-insoluble after the both types of enzyme hydrolysis (scheme II) confirmed the fact that the ^{125}J had been incorporated exclusively into a protein component sedimenting with DNA from H_2O phase. This reduced DNA-peptide complex was used to study its resistance to chemical reagents.

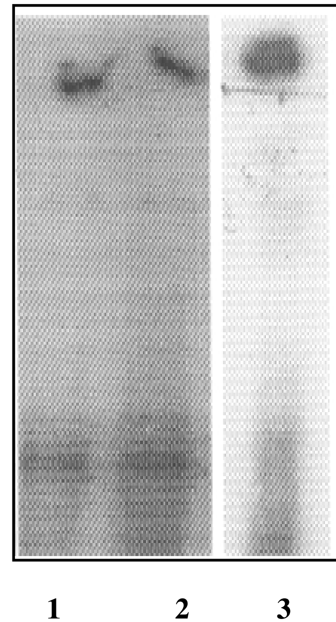


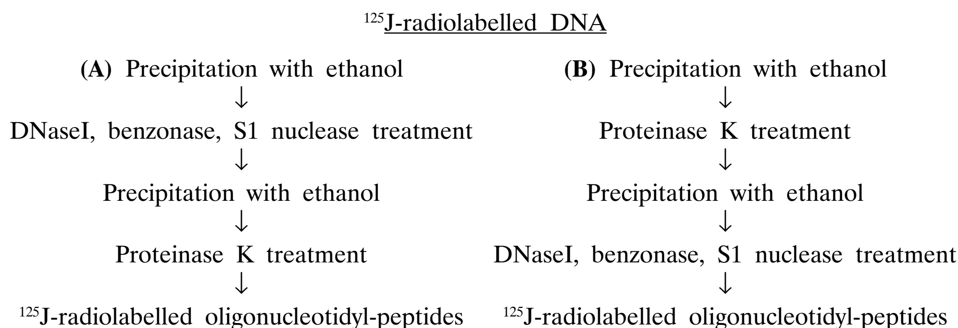
Fig. 1. Autoradiography of DNA-protein complexes from EAT cells. 1 – after extensive digestion with DnazeI; 2 – additionally digested with S1 nuclease; 3 – without nuclease digestion

Theoretically, the residual amino acid /peptide material can be bound to DNA ends by five bonds, which differ in their resistance to acid and alkali [20, 21].

1. Esters of O^4-phospho-tyrosine
2. Esters of N^3-phospho-histidine
3. Esters of phospho serine and phospho-threonine
4. Esters of α-amino-phosphoryl-amino acids (phosphoamidates)
5. Amino acyl esters.

The almost identical peptide hydrolysis profiles isolated from EAT and MEL indicate that a stable DNA-protein complex is not a random association of DNA with some non-histone proteins but a definite, regularly present complex. Its unusual stability could be explained only by a covalent linkage.

As is seen in Table and in Fig. 2, the complex was stable in 0.5 M HCl (2 h, 56 °C), partially alkali-stable (0.1 M NaOH, 2 h, 56 °C). Therefore possible linkages 2, 3, 4, 5 (Table) could be eliminated that are clearly sensitive to these



Scheme 2. Radioiodinated oligonucleotidyl-peptides were isolated by two methods

Table. **Chemical stability of model oligonucleotidyl-peptides**

Type of compound	Alkali (0.1M NaOH, 56 °C, 2 h)	Acid (0.5 M HCl, 56 °C, 2 h)
Radioiodinated EAT oligonucleotidyl-peptides	Partially stable	Stable
Radioiodinated MEL oligonucleotidyl-peptides	Partially stable	Stable
1. Esters of O^4-phospho-tyrosine [22, 23]	Stable	Stable
2. Esters of N^3-phosphohistidine [24]	Stable	Sensitive
3. Esters of phospho serine and phospho-threonine [25, 23]	Sensitive	Stable
4. Esters of α-amino-phosphoryl-amino acids (phosphoamidates) [25, 26]	Stable	Sensitive
5. Amino acyl esters [27]	Sensitive	Sensitive

treatments [20, 21]. These data strongly suggest an ester bond between DNA phosphates and hydroxyamino acid.

These results were confirmed by a release of the bound peptides after treatment of the radioiodinated oligonucleotidyl-peptide complex with phosphodiesterases. In these experiments radioiodinated nucleotidylpeptides (5'-end phosphate groups were removed by alkaline phosphatase) were digested extensively with the spleen phosphodiesterase (0.2 U per 20 µl, overnight at 37 °C) or snake venom phosphodiesterase (0.01 U per 20 µl, overnight at 37 °C), and the hydrolysis products were chromatographed in the phenol/citrate system. PDE I releases 5'-deoxyribonucleotides and cleaves mainly phosphodiesters in the 5'-position between deoxyribonucleotides and nondeoxyribonucleotide material. In contrast, PDE II releases 3'-deoxyribonucleotides from DNA and cleaves preferiantially phosphodiesters in the 3'-position between deoxyribonucleotides and nondeoxyribonucleotide material. PDE I released 40 to 45% of the labeled oligopeptides, while PDE II released

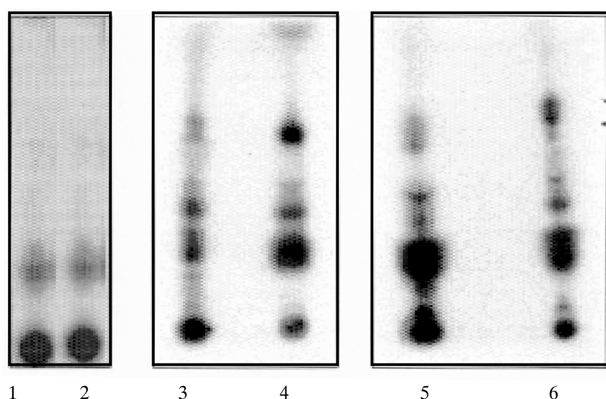


Fig. 2. Autoradiographs of chromatograms after hydrolysis of oligonucleotidyl-peptides with: 2 – 0.5 N HCl; 3 – after digestion with PDE I; 4 – 0.5 N NaOH; 5 – alkaline phosphatase; 6 – PDE II. 1 – oligonucleotidyl peptides incubated in H_2O

up 70% of the total radioactivity (Fig. 2). Several fainted spots not corresponding to the unlabeled standards probably result from incomplete hydrolysis of peptide bonds and/or phosphonucleoside bonds.

The chemical and enzymatic treatment of ^{125}J-treated DNA shows that residual proteinase K resistant peptides, which must be considered to be protease-induced fragments of the polypeptides co-purifying with DNA are bound to 3'-ends and 5'-ends of denatured chromosomal DNA in phosphodiester linkages. The chemical stability of the linking groups and our present knowledge suggest that O^4-phosphotyrosine is an amino acid derivative joining the peptides covalently to DNA.

Our laboratory has accumulated great experience of synthesis of model phosphoamide and phosphoester synthetic derivatives of nucleotides and amino acids. The hydrolytic properties of such model compounds were examined very thoroughly [20–23, 26, 28]. Since radioiodinated oligonucleotidyl-peptides are acid-stable (0.5 N HCl, 2h, 56 °C) and partialy labile in neutral and alkaline medium (0.1 and 0.5 N NaOH, 2h, 56 °C), (Fig. 2), our experience with model compounds allows to conclude that peptide material and DNA are bound by ester bond between DNA phosphates and hydroxyamino acid (Tyr). However there are a few posibilities for tyrosine to bound to DNA:

(i) the internucleotide phosphate
(ii) 5'-phosphate
(iii) 3'-phosphate.

Our experimental data on the chemical stability of oligonucleotidyl-peptide(s) show that natural covalent DNA complexes are phosphotriesters. Radioiodinated oligonucleotidylpeptides demonstrated only partial stability in alkali. This means that 3'-OH and 5'-OH bound phosphotyrosines are absent in the natural complex (these compounds are stable in alkali). We suppose that 3'-OH and 5'-OH bound peptides (data on enzymatic hydrolysis of oligonucleotidyl-peptide(s)) can be produced after partial hydrolysis of internucleotide-bound peptide, which is partially labile in alkali [28].

The present work provides an evidence for the existence of a covalently bound proteins (peptides) in the murine nucleus belonging to matrix (MEL) and DNA-tightly bound proteins (EAT). The nature of such covalently bound complexes is yet to be clarified. Our present goal was to determine specific DNA sequences and the domains responsible for protein binding.

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KOVALENTINIAI DNR BALTYMŲ KOMPLEKSAI IŠ PELIŲ LĄSTELIŲ

S a n t r a u k a

Stipriai deproteinizuota DNR, išskirta iš pelių EAT ir MEL ląstelių, turi nedidelį kiekį tvirtai susirišusių polipeptidų. Tirta šių peptidų prigimtis ir struktūriniai prie DNR prisijungimo aspektai. ^{125}J žymė įvesta į peptido(ų), stipriai susirišusio(ų) su DNR Tyr, His, Arg ir Lys, liekanas. Hidrolizavus DNR baltyminio komplekso nukleotidinę ir peptidinę dalį, išskirtas „mazginis“ oligonukleotidpeptidas, kurio cheminio ir fermentinio stabilumo tyrimai leidžia teigti, kad peptidas(ai) prie DNR yra prisijungęs(ę) per O^4-fosfotiroziną.