ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 4, pp. 454-460. © Pleiades Publishing, Inc., 2006.
Original Russian Text © M. A. Timchenko, E. Yu. Rybalkina, A. Yu. Lomakin, K. I. Evlakov, I. N. Serdyuk, M. G. Ivanovskaya, 2006, published in Biokhimiya, 2006, Vol. 71, No. 4, pp. 561-569.

Modified DNA Fragments Specifically and Irreversibly Bind Transcription Factor NF-κB in Lysates of Human Tumor Cells

M. A. Timchenko[1]*, E. Yu. Rybalkina[2], A. Yu. Lomakin[3], K. I. Evlakov[3], I. N. Serdyuk[3], and M. G. Ivanovskaya[1]

[1]Belozersky Institute of Physico-Chemical Biology and Faculty of Chemistry, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-3181; E-mail: img@genebee.msu.su; mtimchenko@vega.protres.ru
[2]Institute of Carcinogenesis, Blokhin Cancer Research Center, Russian Academy of Medical Sciences, 115478 Moscow, Russia; fax: (495) 324-1205; E-mail: rybalkina@crc.umos.ru
[3]Institute of Protein Research, 142290 Pushchino, Moscow Region, Russia; fax: (495) 924-0493; E-mail: serdyuk@vega.protres.ru

Received June 15, 2005
Revision received September 15, 2005

Abstract—Covalent binding of a synthetic DNA fragment with eukaryotic transcription factor NF-κB has been studied in lysates of human colon carcinoma HCT-116 cells. For binding we used ^{32}P-labeled 17/19 bp nucleotide DNA duplex containing an NF-κB recognition site (κB-site) in which one of internucleotide phosphate groups was replaced by a chemically active trisubstituted pyrophosphate group. Using gel electrophoresis under denaturing conditions (Laemmli electrophoresis) followed by immunoblotting revealed selective irreversible binding of ^{32}P-labeled DNA duplex with NF-κB in lysates of tumor cells in the presence of other cell components. Experiment on delivery of this DNA duplex containing rhodamine at 3′-end of the modified chain in an intact cell revealed that rhodamine-labeled DNA penetrated through the plasma membrane of tumor cells without any additional delivery systems. Using fluorescent microscopy, we found that the rhodamine-labeled DNA is initially localized in the cytoplasm. Confocal laser scanning microscopy revealed that subsequent treatment of the cells with TNF-α promoted partial translocation of the DNA reagent into the nucleus.

DOI: 10.1134/S0006297906040158

Key words: transcription factor NF-κB, modified DNA duplexes, covalent binding, DNA–protein interactions

Studies of molecular mechanisms of genetic processes have revealed the crucial role of protein factors in regulation of gene expression on various levels. The nuclear transcription factor NF-κB is one of the key eukaryotic transcription factors. This protein consists of two subunits: p50 of 50 kD and p65 of 65 kD [1]; NF-κB can exist as the p50-p65 heterodimer or p50-p50 and p65-p65 homodimers. The subunit p50 is responsible for DNA binding, whereas p65 activates transcription [2]. In mammalian cells (except B cells) NF-κB exists in cytoplasm as an inactive complex with inhibitory protein IκB; the latter shields a nuclear localization signal of NF-κB responsible for transport of active NF-κB from the cytoplasm into the nucleus. The action of cytokines (e.g., TNF-α), viruses, bacteria, ultraviolet irradiation, and reactive oxygen species on these cells leads to NF-κB activation. This activation involves a complex of kinases phosphorylating IκB, which then undergoes ubiquitinylation followed by proteolysis with 26S proteasome. Active NF-κB is translocated into the nucleus where it forms a specific complex with a certain decanucleotide sequence of chromosomal DNA known as the κB site. This initiates transcription of genes encoding various proteins including signal and immune response proteins [3]. Studies of molecular mechanisms underlying tumor cell transformation, multiple drug resistance of tumor cells, and apoptosis revealed an important role of the transcription factor NF-κB in the development of these phenomena. Tumor cells are characterized by high expression of NF-κB, which triggers a cascade reactions leading to activa-

Abbreviations: CLSM) confocal laser scanning microscopy; EDC) 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide; HCT-116) human colon carcinoma cell line; NF-κB) nuclear factor of immunoglobulin κ light chain enhancer in B cells; PMSF) phenylmethylsulfonyl fluoride; TNF-α) tumor necrosis factor α; TSPG) trisubstituted pyrophosphate group.

* To whom correspondence should be addressed.

tion of oncogene transcription [4]. This explains the intensive search for effective pathways for modulation of NF-κB activity in human tumor cells.

One promising approach for regulation of transcription factor activity consists in the use of synthetic DNA molecules containing a protein recognition site. These DNAs bind such protein and therefore inhibit its interaction with chromosomal DNA [5]. In the present study, we have used synthetic DNA fragments containing κB site as "molecular decoys". The κB site in these DNA decoys was modified by introduction of a trisubstituted pyrophosphate group (TSPG). (We have described the approach for design and synthesis of such "decoys" earlier [6].) DNA duplexes containing in the required position of the sugar-phosphate backbone the chemically active TSPG instead of the natural internucleotide phosphodiester bonds [7] are effective phosphorylating agents. In aqueous solution they can react with nucleophilic groups of amino acid residues (Lys or His) of proteins with covalent bond formation. The formation of the covalent bond between DNA and protein requires steric closeness of reactants, which is realized only in the case of specific complex formation between DNA and a protein [6]. Recognition and binding of modified DNA reagents with protein is not only selective but also irreversible. Selectivity of such interactions is achieved by the presence of a protein recognition site in DNA and irreversibility is determined by covalent bond formation.

In this study, we have investigated the interaction of mentioned type of reagents with NF-κB protein in human colon carcinoma HCT-116 cells. The major task of this study was to demonstrate selectivity of interaction of TSPG-containing DNA reagent with its target, NF-κB protein in HCT-116 cells. Selectivity of interaction was investigated in lysates of these cells. Using ^{32}P-labeled DNA duplex and the method of gel electrophoresis under denaturing conditions, we have demonstrated covalent DNA–protein complex formation. These complexes containing proteins of 50, 65, and 90 kD were detected in nuclear fraction of the cell lysate; some traces of the complex between DNA and protein of 50 kD were also detected in the cytoplasmic fraction. Subsequent analysis of covalently bound products in the nuclear lysate fraction by immunoblotting with antibodies against p50 and p65 subunits of NF-κB revealed that the TSPG-containing DNA duplex selectively and irreversible bound NF-κB in the nuclear fraction of HCT-116 cell lysates. In this study, we have also analyzed delivery of this DNA reagent (containing a tetramethylrhodamine at its 3′-end) into HCT-116 tumor cells.

MATERIALS AND METHODS

The p50-subunit of NF-κB transcription factor with native amino acid sequence was isolated from *E. coli* BL21 (DE3) cells transformed with pEt-14b plasmid [8]. This subunit is a conjugate with a 19-membered amino acid sequence of 2 kD, which contains a block of six His residues, and has total molecular weight of 44.5 kD.

Oligodeoxyribonucleotides used in this study were synthesized by the amidophosphite method using an automated synthesizer (Applied Biosystems, USA) and standard reagents (Pharmacia, Sweden). For preparation of fluorescently labeled DNA reagent the residue of tetramethylrhodamine-5-isothiocyanate (5-TRITC, G-isomer) was covalently attached to the 3′-phosphorylated oligonucleotide end by the standard method [9].

The following chemicals were used in this study: Hepes, EDTA, $LiClO_4$, SDS (Fluka, Switzerland); $MgCl_2$, NaCl, Mes, dithiothreitol (DTT) (Merck, Germany); Tris, 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide (EDC), Hoechst 33258 (Sigma, USA); acrylamide, N,N′-methylene-bis-acrylamide, $(NH_4)_2S_2O_8$ (Amresco, USA); N,N,N′,N′-tetramethylethylenediamine, bromophenol blue (Reanal, Hungary); glycerol (ICN, USA); DMEM medium (PanEco, Russia); fetal bovine serum (FBS) (HyClone, USA); gentamicin (Chemopharm, Yugoslavia); Hybond-C nitrocellulose membrane (Amersham, UK); rabbit polyclonal antibodies against the C-terminal part of the p65 subunit of NF-κB (C-20) and N-terminal part (residues 120-239) of the p50 subunit of NF-κB (H-119) (Santa-Cruz Biotechnology, USA); *para*-formaldehyde (Khimmed, Russia); TNF-α (Calbiochem, Germany); 5-TRITC (Molecular Probes, USA).

The radioactive label was introduced into oligonucleotides using [γ-^{32}P]ATP (1000 Ci/mmol; Isotope, Russia) and T4-polynucleotide kinase (25,000 U/ml; SibEnzyme, Russia). Electrophoresis of ^{32}P-labeled oligonucleotides was carried out in plane (25 × 20 × 0.05 cm) polyacrylamide gel (20% acrylamide, 1% N,N′-methylene-bis-acrylamide, 7 M urea in 50 mM Tris-borate buffer (0.05 M Tris, 0.05 M H_3BO_3, and 1 mM EDTA, pH 8.3)) at field strength 60 V/cm. The position of oligonucleotide fragments was determined by autoradiography, and radioactivity of ^{32}P-labeled preparations was determined using a Delta-300 counter (Tracor, Holland). The counting error did not exceed 2%. Oligonucleotides were extracted from the gel using 2 M aqueous solution of $LiClO_4$ for 12 h at 4°C.

Penetration of fluorescent-labeled DNA reagent through the cell membrane and its intracellular localization were studied using an AxioCam fluorescence microscope (Carl Zeiss, Germany) and LSM 510 confocal laser scanning microscope (Carl Zeiss). Rhodamine fluorescence was excited using a He-Ne laser with wavelength $\lambda = 543$ nm. Cell images were obtained using Axioplan2 Imaging and Zeiss LSM Image Browser (version 3.0) programs.

Chemical ligation with formation of TSPG-oligonucleotide derivatives. For chemical ligation, we initially

synthesized the 3′-O-methyl ester of the oligonucleotide by condensation of 3′-phosphorylated oligonucleotide (1-10 nM) with 20% aqueous methanol solution in the presence of EDC (1 mg per 10 μl of reaction mixture) in Mes buffer (0.5 M Mes, 1 M $MgCl_2$, pH 4.5). The total volume of the reaction mixture was 40 μl. The reaction mixtures were incubated for 12 h at 4°C and mixed with 150 μl of 2 M $LiClO_4$. Nucleotide material was precipitated by adding 5-fold excess of acetone (1 ml). After cooling of the resulting mixture to −20°C and incubation for 3 h, the mixture was centrifuged and the pellet was dissolved in water.

For preparation of modified DNAs, the DNA duplex was formed using equimolar quantities of three components: DNA template, 3′-O-methyl oligonucleotide ester, and 5′-phosphorylated oligonucleotide (duplex concentration was 10^{-3}-10^{-4} M); 0.5 M EDC was added in Mes buffer (0.05 M Mes and 0.02 M $MgCl_2$, pH 6.0) [7]. The total volume of the reaction mixture was 20 μl. The reaction mixtures incubated at 4°C for 72 h were then mixed with 150 μl of 2 M $LiClO_4$. The nucleotides material was precipitated by adding 5-fold excess of acetone (1 ml). Modified oligonucleotides separated in 20% polyacrylamide gel in the presence of 7 M urea at 60 V/cm were then identified by autoradiography. Gel strips corresponding to modified oligonucleotides were excised and eluted using 2 M $LiClO_4$. DNA duplexes containing TSPG were formed by adding an equimolar quantity of DNA template to modified oligonucleotide.

Cell cultivation. Human colon carcinoma HCT-116 cells were used in this study. Cells were cultivated at 37°C under an atmosphere of 5% CO_2 in DMEM medium containing 10% FBS and 50 μg/ml gentamicin. In experiments we used only cell cultures reaching subconfluent state.

Isolation of nuclear and cytoplasmic lysates. For activation of NF-κB, cell cultures were incubated with TNF-α (final concentration 30 ng/ml) for 1 h, washed three times with 0.05 M sodium phosphate buffer (pH 7.3), and precipitated by centrifugation. The cell precipitate ($3 \cdot 10^6$) was resuspended in four volumes (100 μl) of HB buffer (25 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 5 mM KCl, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and incubated in an ice bath for 5 min. After addition of 10% Nonidet P-40 (NP-40) to final concentration 0.5%, the mixture was incubated in the ice bath for 15 min for lysis of cells and release of the nuclei. The nuclei were precipitated by centrifugation at 1500*g* for 10 min. The supernatant containing the fraction of cytoplasmic proteins was placed into another tube and used in subsequent analyses. The precipitate of cell nuclei was resuspended in 20 μl of NB buffer (20 mM Tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 400 mM NaCl, 1 mM PMSF, 0.1% NP-40) and incubated in the ice bath for 15 min. The nucleic acid fraction was precipitated by centrifugation (13,000*g*, 15 min). The supernatant containing the fraction of nuclear proteins was placed into another tube and used for subsequent analysis. Protein content in nuclear and cytoplasmic fractions was determined by the Bradford method [10].

Covalent binding of modified DNA duplexes with NF-κB. a) *Binding with recombinant p50 protein.* Covalent binding with p50 protein was carried out at the protein/DNA ratio 130 : 1. ^{32}P-Labeled modified DNA duplexes (0.3 pmol) were mixed with 43 pmol of p50 protein in the binding buffer (7.5 mM Hepes, pH 8.0, 1 mM $MgCl_2$, 34 mM NaCl, 0.5 mM DTT, and 0.05 mM EDTA) [8]. The total volume of the reaction mixture was 10 μl. The reaction mixture was incubated at 20°C for 12 h. Products of DNA–protein covalent binding were analyzed in 12% SDS-polyacrylamide gel with a 4% concentrating band according to Laemmli [11] using field strength 15 V/cm. Before gel loading samples were mixed with buffer containing 0.06 M Tris-HCl, pH 6.8, 7 M urea, 0.1% glycerol, 2% SDS, 0.05% β-mercaptoethanol, and 0.1% bromophenol blue and incubated at 95°C for 10 min**.** After electrophoretic separation, gels were dried and scanned using a PhosphorImager SI (Molecular Dynamics). Radioactivity of bands corresponding to the complex formed between DNA duplex and transcription factor and also bands corresponding to free duplex were calculated using the ImageQuant 5.0 program. The effectiveness of covalent binding (%) was determined as the ratio of band radioactivity of the complex to total radioactivity of complex and free DNA.

b) *Binding with NF-κB in cell lysates.* For covalent binding of modified DNA duplexes with cell lysate NF-κB, nuclear or cytoplasmic lysates (5 μl, total protein concentration 1.7 mg/ml) were mixed with 0.3 pmol of ^{32}P-labeled DNA duplexes. The covalent binding was carried out at 20°C for 12 h in 10 μl of binding buffer containing 7.5 mM Hepes, pH 8.0, 1 mM $MgCl_2$, 34 mM NaCl, 0.5 mM DTT, and 0.05 mM EDTA. Products of covalent binding were analyzed in 12% SDS-polyacrylamide gel as described above for p50 protein.

Immunoblotting. The products of covalent binding of DNA duplex and nuclear extract proteins were separated in 12% SDS-polyacrylamide gel by the method of Laemmli [11]. After electrophoresis, the proteins were transferred onto Hybond-C nitrocellulose membrane using a MiniTrans-Blot (BioRad, USA). Buffer and transfer conditions were recommended by the suppliers. Immunoblotting was carried out using the ECL Western blotting protocol (Amersham). The membrane was washed with TBST buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) for 20 min; nonspecific sorption was blocked by incubation for 1 h with 5% defatted dry milk diluted in TBST.

For identification of the transcription factor NF-κB in nuclear and cytoplasmic lysates, we used rabbit polyclonal antibodies to the C-terminal part of the NF-κB p65 subunit and the N-terminal part of the NF-κB p50

subunit (residues 120-239). Antibodies elaborated against rabbit immunoglobulins and conjugated with horseradish peroxidase (AP132F; Chemicon, USA) were used as the second antibodies. For signal detection on immunoblotting, we used the ECL system with the following substrates: 1.25 mM luminol, 0.2 mM *p*-coumaric acid, and 0.01% H_2O_2 in 150 mM Tris-HCl, pH 8.8. Chemiluminescence was registered using an X-ray film.

Preparation of cell samples for fluorescent and confocal laser scanning microscopy (CLSM). HCT-116 cells were cultivated under standard conditions on slides placed in Petri dishes (40 mm). Cells reaching the subconfluent state were incubated with rhodamine-labeled DNA reagent (B) (final concentration 10 pmol/μl) at 37°C for 3 h and then washed with 0.05 M sodium phosphate buffer (pH 7.3). For preparation of temporal samples of living cells, the slides with treated cells were immobilized on a glass bed using 50% glycerol in 0.05 M sodium phosphate buffer. For visualization of nuclei, cells were stained with Hoechst solution before addition of glycerol. These preparations were analyzed using a fluorescence microscope. For analysis of cells by means of CLSM, cells treated with the rhodamine-labeled DNA reagent were fixed using 4% solution of *p*-formaldehyde and put into Elvanol.

Transport of DNA reagent into cell nuclei was studied in HCT-116 cells prepared as described above. Cells were incubated with DNA at 37°C for 3 h and then were treated with TNF-α (final 30 ng/ml) for 1 h at 37°C. The cells were then washed with 0.05 M sodium phosphate buffer (pH 7.3) and prepared for analysis by means of fluorescence microscopy and CLSM as described above.

The pEt-14b plasmid carrying the gene encoding NF-κB transcription factor p50 subunit was kindly supplied by Professor A. Israel (Pasteur Institute, France).

The rhodamine-labeled oligonucleotide was synthesized and kindly presented by Dr. M. Volkov (Faculty of Chemistry, Lomonosov Moscow State University).

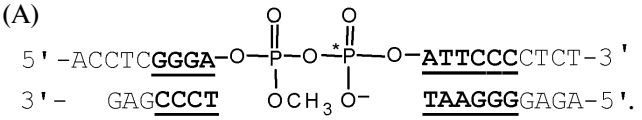
Initial oligonucleotides were synthesized and kindly presented by Dr. E. A. Romanova (Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University).

Studies of cell samples by means of CLSM were carried out together with Dr. V. A. Yashin (Institute of Theoretical and Experimental Biophysics, Pushchino, Russia).

RESULTS

Analysis of covalent binding specificity between modified DNA duplex and NF-κB in tumor cells. To test the specificity of DNA fragment binding with NF-κB in tumor cells, we have used ^{32}P-labeled DNA duplex of the following structure (duplex A):

(A)



DNA duplex A was synthesized by chemical ligation as described in [7]. TSPG was inserted into one of the selected positions of the idealized palindrome κB-site of DNA [1]. Our pilot experiments revealed that duplex A exhibits the most effective binding of NF-κB p50 protein *in vitro* (up to 50%).

Binding specificity of DNA duplex A, containing TSPG at the fourth position of the κB-site, with NF-κB protein was analyzed in lysates of eukaryotic cells. Human colon carcinoma HCT-116 cells pretreated with TNF-α were used for preparation of nuclear and cytoplasmic fraction of cell lysates. These fractions and also recombinant p50 protein of 44.5 kD used as a marker were incubated with ^{32}P-labeled DNA duplex A under optimal conditions favoring interaction of TSPG containing DNA duplex with NF-κB p50 protein (20°C, 12 h). Products of covalent binding were analyzed by gel electrophoresis under denaturing conditions (12% SDS-polyacrylamide gel, Laemmli electrophoresis) followed by autoradiography. Figure 1 shows the gel autoradiograph. These results indicate that three tightly bound DNA–protein complexes are formed in nuclear lysate.

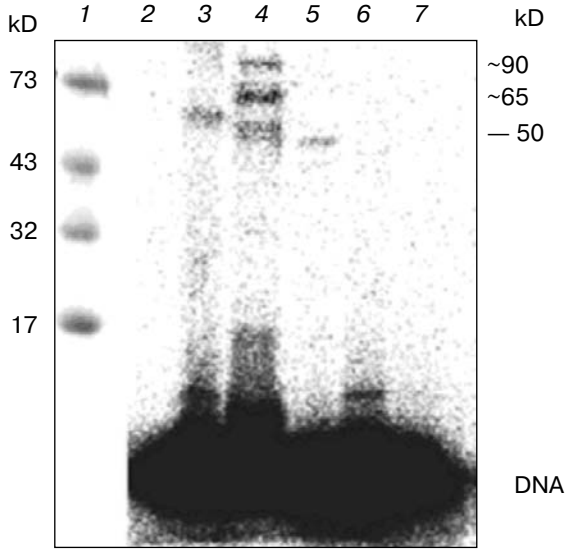


Fig. 1. Autoradiograph of protein conjugates with DNA duplexes in 12% SDS-polyacrylamide gel. Lanes: *1*) molecular weight protein markers (stained with Coomassie G-250); *2*, *6*, *7*) products of covalent binding of nonspecific DNA duplex B with recombinant p50 protein (44.5 kD), nuclear and cytoplasmic fractions of HCT-116 cell lysate, respectively; *3-5*) products of covalent binding of specific DNA duplex A with recombinant p50 protein (44.5 kD) and nuclear and cytoplasmic fractions of HCT-116 cell lysate, respectively. The label (^{32}P) was at TSPG instead of phosphate group of disubstituted phosphate.

These complexes apparently contain proteins p50 (50 kD), p65 (65 kD), and the ubiquitinylated form of p50 (~90 kD). In cytoplasmic fraction formation of only one covalent conjugate between DNA duplex A and p50 protein was observed.

^{32}P-Labeled DNA duplex B lacking NF-κB protein recognition site but carrying TSPG was used as a control for specificity:

(B)

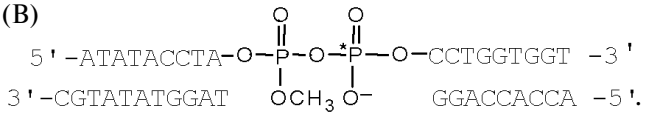


Figure 1 shows that nonspecific DNA duplex B does not form covalent complexes with either recombinant p50 protein or proteins of the nuclear and cytoplasmic fractions of cell lysates.

For elucidation in nuclear and cytoplasmic fractions of various NF-κB subunits covalently bound to DNA duplex, we employed immunoblotting with rabbit polyclonal antibodies to NF-κB p65 and p50 subunits. Results of immunoblotting with antibodies against NF-κB p50 subunit (Fig. 2, lane *2*) illustrate the presence of p50 protein and probably its ubiquitinylated form (~90 kD) in the nuclear fraction of HCT-116 cell lysate. Immunoblotting with antibodies to NF-κB p65 (Fig. 2, lane *3*) also revealed the presence of NF-κB p65 subunit in this lysate. Results of immunoblotting (Fig. 2) indicate that bands on the autoradiograph (Fig. 1, lane *4*) related to the covalent DNA–protein conjugates in the nuclear fraction of cell lysate correspond to NF-κB p50 and p65 subunits and possibly to a ubiquitinylated form of p50 [12]. Nonspecific binding of DNA duplex A with other lysate proteins was not detected. Consequently, specific DNA duplex A containing TSPG in its protein recognition site selectively binds NF-κB.

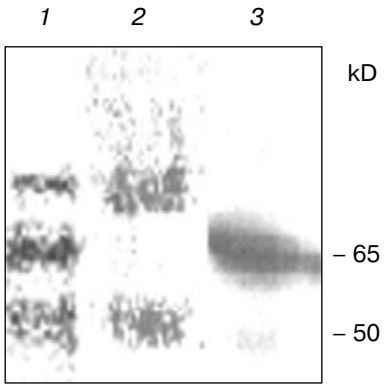
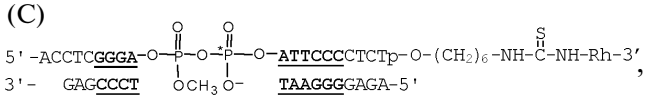


Fig. 2. Immunoblotting of proteins covalently bound to specific DNA duplex A in nuclear fraction of HCT-116 cell lysate. Lanes: *1*) autoradiograph of proteins covalently bound to specific DNA duplex A in nuclear fraction of HCT-116 cell lysate (see Fig. 1, lane *4*); *2*) immunoblotting of covalently bound proteins with antibodies to NF-κB p50; *3*) immunoblotting of covalently bound proteins with antibodies to NF-κB p65.

Study of penetration and intracellular localization of rhodamine-labeled DNA reagent in intact HCT-116 cells by means of fluorescence and confocal laser scanning microscopy. For investigation of penetration of DNA reagent through the plasma membrane of tumor cells, we have synthesized TSPG-containing DNA duplex (C) carrying a covalently bound rhodamine residue at the 3′-end of the modified chain:

(C)



where Rh represents tetramethylrhodamine residue.

HCT-116 cells were incubated with DNA duplex C for 3 h and intracellular distribution of the DNA reagent was analyzed by means of fluorescence and confocal microscopy. These experiments revealed that this DNA reagent penetrates through the plasma membrane without using any delivery system and this reagent is localized in the cytoplasm (Fig. 3). It is possible that the presence of the covalently bound hydrophobic rhodamine residue at the 3′-end facilitates transmembrane penetration of this DNA duplex [13].

Gel electrophoresis under denaturing conditions in 20% polyacrylamide gel revealed that 3-h incubation of rhodamine-labeled DNA duplex with cytoplasmic fraction of HCT-116 cell lysate was not accompanied by detectable duplex degradation. Duplex stability to nuclease degradation is determined by double-helical structure of the DNA reagent [14], the presence of the rhodamine residue at the 3′-end of oligonucleotide, and the compact structure of DNA-protein complex [15].

For evaluation of possible subsequent translocation of the DNA reagent from the cytoplasm into the nucleus, HCT-116 cells preincubated with rhodamine-labeled DNA duplex C were then treated with TNF-α (for activation of NF-κB) for 1 h. The resulting cell samples were analyzed using fluorescence microscopy and CLSM. These experiments revealed that cell treatment with TNF-α was accompanied by partial migration of the DNA reagent into the nucleus possibly due to covalent and noncovalent binding of the DNA reagent with TNF-α-activated NF-κB (Fig. 4).

DISCUSSION

Development of drugs exhibiting not only high efficacy but also selectivity is one of the main goals of modern pharmacology and chemotherapy. Such therapeutic agents may be constructed on the basis of natural biopoly-

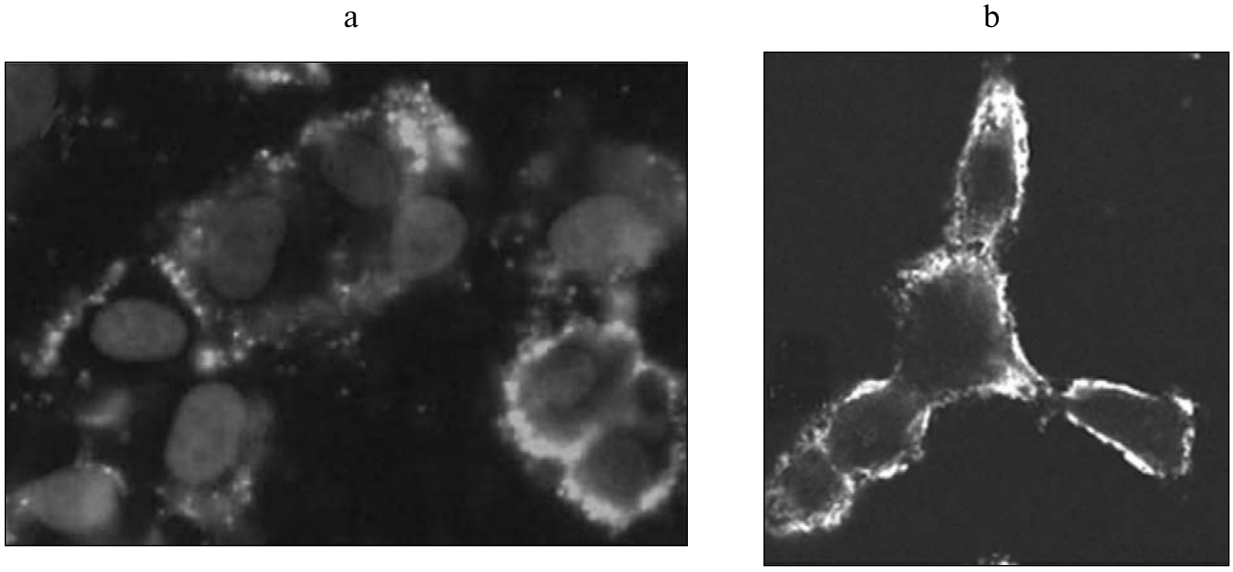


Fig. 3. Photographs of HCT-116 cells incubated with rhodamine-labeled DNA duplex C obtained using fluorescence microscopy (a) (nuclei were stained with Hoechst) and CLSM (b). In the cell cytoplasm the fluorescence of rhodamine-labeled DNA is observed.

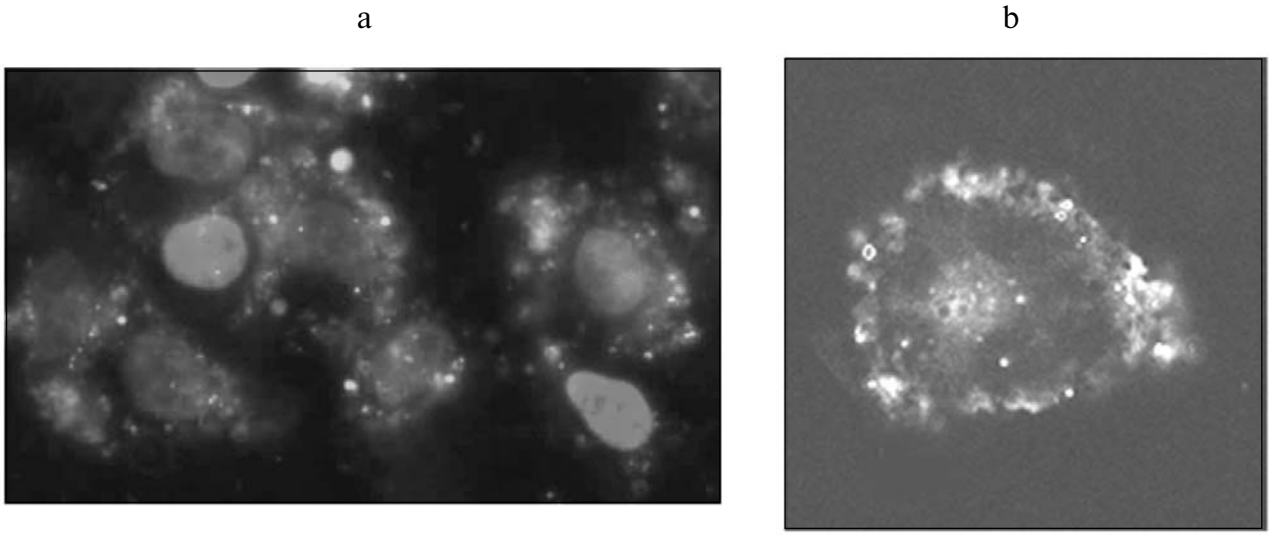


Fig. 4. Photographs of HCT-116 cells incubated with rhodamine-labeled DNA duplex C and activated by TNF-α: a) obtained by fluorescence microscopy (nuclei were stained with Hoechst); b) obtained by CLSM. Fluorescence of rhodamine-labeled DNA is observed in the cytoplasm and nucleus.

mers; selectivity of their effects is determined by biospecific delivery of such biopolymers and their binding with certain cell compartments.

Design of DNA "decoys" based on synthetic DNA fragments is one of the most promising approaches for development of drugs. Such "decoys" would selectively interact with transcription factors and inhibit oncogene expression in the cell at transcription level. Taking into consideration that NF-κB activates transcription of antiapoptotic genes, its selective inhibition is an important problem for medicine.

In this study we have demonstrated that modified DNA duplex containing chemically active trisubstituted pyrophosphate group in the fourth position of the κB recognition site of the eukaryotic transcription factor NF-κB can effectively and selectively bind this protein in cell lysates of tumor HCT-116 cells in the presence of all cell components. Using fluorescence microscopy and CLSM, we demonstrated that during 3-h incubation fluorescently labeled DNA duplex (10^{-5} M) penetrates through the plasma membrane of HCT-116 cells and is localized in the cytoplasm. CLSM revealed that subse-

quent treatment of these cells with TNF-α, which activates NF-κB, resulted in partial translocation of the fluorescently labeled DNA reagent from cytoplasm into the cell nucleus. Since activation of NF-κB by TNF-α may occur only in cytoplasm [16], transport of the DNA reagent through nuclear membrane requires complex formation with activated NF-κB. However, in this study we could not discriminate possible covalent and noncovalent complexes between DNA duplex and NF-κB in a cell.

The approach that we have proposed for inhibition of activity of the transcription factor NF-κB is based on a rather simple and inexpensive method of tight and selective covalent binding between NF-κB and modified DNA duplexes. We believe that such DNA duplexes are a promising basis for design of nontoxic therapeutic agents that inhibit cell proliferation and tumorigenesis.

Our data indicate possible use of the synthesized duplexes for detection of active transcription factor NF-κB, which can bind to its recognition site on the DNA molecule and participate in the process of initiation of transcription. Since many inflammatory and tumor diseases are characterized by high level of NF-κB expression, the evaluation of the active form of this transcription factor in various cells and comparison with its content in normal healthy cells can detect deviations from normal status of the cell.

This work was supported by the Russian Foundation for Basic Research (grant Nos. 02-03-32950-a and 04-04-97255-p2004naukograd_a).

REFERENCES

1. Ghosh, G., Duyne, G., Ghosh, S., and Sigler, P. (1995) *Nature*, **373**, 303-310.
2. Caamano, J., and Hunter, C. A. (2002) *Clin. Microbiol. Rev.*, **15**, 414-429.
3. Viatour, P., Merville, M.-P., Bours, V., and Chariot, A. (2005) *Trends Biochem. Sci.*, **30**, 43-51.
4. Perkins, N. D. (2004) *Trends Cell Biol.*, **14**, 64-69.
5. Wu, H., Holcenberg, J. S., Tomich, J., Chen, J., Jones, P. A., Huang, S. H., and Calame, K. L. (1990) *Gene*, **89**, 203-209.
6. Kozlov, I. A., Kubareva, E. A., Ivanovskaya, M. G., and Shabarova, Z. A. (1997) *Antisense Nucleic Acid Drug Dev.*, **7**, 279-289.
7. Kuznetsova, S. A., Ivanovskaya, M. G., and Shabarova, Z. A. (1990) *Bioorg. Khim.*, **16**, 219-225.
8. Ljungquist, C., Breitholtz, A., Brink-Nilsson, H., Moks, T., Uhlen, M., and Nilsson, B. (1989) *Eur. J. Biochem.*, **186**, 563-569.
9. Agrawal, S., Christodoulou, C., and Gait, M. J. (1986) *Nucleic Acids Res.*, **14**, 6227-6245.
10. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
11. Laemmli, U. (1970) *Nature*, **227**, 680-685.
12. Ivanova, T. V., Ivanov, V. N., and Nadezhdina, E. S. (2001) *Membr. Cell Biol.*, **14**, 727-741.
13. Neyfakh, A. A. (1988) *Exp. Cell. Res.*, **174**, 168-178.
14. Yanze, M. F., Lee, W.-S., Poon, K., Piquette-Miller, M., and Macgregor, R. B., Jr. (2003) *Biochemistry*, **42**, 11427-11433.
15. Tam, R. C., Ying, L., Noonbergwang, D. G., Lui, G., Hunt, C. A., and Garovoy, M. R. (1994) *Nucleic Acids Res.*, **22**, 6977-6986.
16. Sebald, A., Mattioli, I., and Schmitz, M. L. (2005) *Eur. J. Immunol.*, **35**, 318-325.