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Photoactivated DNA Analogs of Substrates of the Nucleotide Excision Repair System and Their Interaction with Proteins of NER-Competent Extract of HeLa Cells. Synthesis and Application of Long Model DNA

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Abstract—Long linear DNA analogs of nucleotide excision repair (NER) substrates have been synthesized. They are 137-mer duplexes containing in their internal positions nucleotides with bulky substitutes imitating lesions with fluorochloroazidopyridyl and fluorescein groups introduced using spacer fragments at the 4N and 5C positions of dCMP and dUMP (Fap-dC- and Flu-dU-DNA) and DNA containing a (+)-*cis*-stereoisomer of benzo[a]pyrene-*N2*-deoxyguanidine (BP-dG-DNA, 131 bp). The interaction of the modified DNA duplexes with the proteins of NER-competent HeLa extract was investigated. The substrate properties of the model DNA in the reaction of specific excision were shown to vary in the series Fap-dC-DNA << Flu-dU-DNA < BP-dG-DNA. During the experiments on affinity modification of the proteins of NER-competent extract, Fap-dC-DNA (137 bp) containing a ^{32}P-label in the photoactive nucleotide demonstrated properties of a highly efficient and selective probe. The set of the main targets of labeling included polypeptides of the extract with the same values of apparent molecular weights (35-90 kDa) as when using the shorter (48 bp) Fap-dC-DNA. Besides, some of the extract proteins were shown capable of specific and effective interaction with the long analog of NER substrate. Electrophoretic mobility of these proteins coincided with the mobilities of DNA-binding subunits of XPC-HR23B and PARP1 (~127 and ~115 kDa, respectively). The 115-kDa target protein was identified as PARP1 using NAD^+-based functional testing. The results suggest that the linear Fap-dC-DNA is an unrepairable substrate analog that can compete with effective NER substrates in the binding of the proteins responsible for lesion recognition and excision.

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Key words: long bulky substituted DNA duplexes, protein factors of NER preincision complexes, photoaffinity modification

In eukaryotic cells, nucleotide excision repair (NER) in DNA is performed through coordinated action of multicomponent protein complexes of variable composition that are sequentially assembled in the damaged DNA region. The main (core) NER enzymes and protein factors have been identified, but the architecture of specific protein–nucleic acid complexes, the mechanism of their functioning, and interaction of their components are still subjects of active investigation. DNA lesions repaired by NER are usually the products of modification of nitrogen bases: three-dimensional adducts disturbing the regular double-helical structure of the DNA molecule. Such lesions appear as a result of exposure of DNA to various physical or chemical factors (UV or ionizing radiation, environmental pollutants, chemotherapeutic agents, chemically active substances appearing in an organism during chronic inflammation, etc.). During NER in a eukaryotic system, the region with the damaged link (24-32 nucleotides) is removed from the DNA followed by reconstruction of the nucleotide sequence using the undamaged DNA chain as a template for the reparative synthesis [1-3].

Abbreviations: BP-dG, (+)-*cis*-benzo[a]pyrene-*N2*-deoxyguanine; Fap-dCTP, exo-*N*-{2-[N-(4-azido-2,5-difluoro-3-chloropyridin-6-yl)-3-aminopropionyl]aminoethyl}-2′-deoxycytidine-5′-triphosphate; HR23B, 58-kDa polypeptide, a minor subunit of functional heterodimer XPC-HR23B; NER, nucleotide excision repair; ONT, deoxyoligonucleotide; PARP1, poly-(ADP-ribose)-polymerase-1; RPA, human replication A factor; XPC, *xeroderma pigmentosum* complementation group C factor.

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Many biochemical approaches to NER investigation are based on synthetic substrate analogs, i.e. double-stranded DNA bearing three-dimensional modification in the target position of the molecule, and structural analogs of the intermediates that appear during NER [4-9]. This fact determines the interest in development and improvement of methods of synthesis of model lesions and creation of synthetic DNA on their basis [10-12]. Photoactive arylazide, including 4-azide-2,5-difluoro-3-pyridin-6-yl (Fap), and fluorescent fluorescein (Flu) and anthracenyl groups, introduced at the C5-or N4-position of pyrimidine nitrogen bases via linker fragments were proposed in our previous works [13-17] for modeling DNA lesions caused by the effect of aromatic hydrocarbons and their derivatives. They were used to create a broad spectrum of NER substrate analogs. Sets of modified DNA duplexes of 48 and 60 bp synthesized by a chemical-enzymatic method were used to study the binding of NER proteins to damaged DNA using the gel retardation assay and measurement of the quantitative characteristics of protein–nuclein interaction by equilibrium fluorescence titration [13, 14, 16]. The experiments on affinity modification of NER proteins in reconstructed systems of different degrees of complexity with application of 48- and 60-bp photoactive DNA duplexes revealed the nature of mutual influence of the XPC, RPA, and XPA factors on their interaction with damaged DNA [13-19].

NER is characterized by broad substrate specificity. However, the rates of repair of structurally similar lesions can be substantially different. The efficiency of removal of the DNA region with a damaged nucleotide estimated using model substrates, in combination with the results of analysis of interaction between substrate analogs and NER proteins, provides information on the details of lesion recognition by the NER system. It is known, however, that a model lesion must be included in long DNA for the formation of functional NER complexes. In some works, substrate properties were studied using ring structures based on a phage or plasmid DNA and bearing lesions in the target position (or positions) or statistically introduced into the DNA molecule at a particular frequency. The substrate properties of Fap-dC (-dU) were estimated also with application of ring DNA based on plasmid pBSK II [17].

Another type of model structures being substrates of the eukaryotic NER system is linear DNA duplexes of >120 bp in length bearing a modified nucleotide in an internal position of the chain at approximately equal distances from the ends of the duplex [20]. Such DNA are universal models applicable both for experiments on the estimation of substrate properties of lesions and for creation of affinity probes of different structure. In particular, the use of long DNA analogs of substrates containing regions of several unpaired bases, including those introduced at a distance from the lesion, made it possible to specify the role of the undamaged chain in lesion recognition and to confirm the hypothesis of a two-stage (or bipartite) [8, 21] mechanism of lesion recognition. For instance, it has been shown that the efficiency of repair of model DNA containing hard-to-repair cyclobutane pyrimidine dimers noticeably increases on introduction of a bubble or a loop of three unpaired bases in size on the 5′-side of the lesion [21]. Besides, it was recently shown by the example of long linear DNA containing a benz[a]pyrene modification that lesion recognition by NER proteins and efficiency of lesion removal depend not only on the type of modification introduced but also on details such as differences in the spatial structure of the lesion [22] and sequence of the regions flanking the lesion [23].

The goal of the present work was to create long linear DNA analogs of NER substrates and then to analyze their interaction with proteins of cell extracts containing a complex of NER proteins and to estimate the properties of the created model DNA as substrates of the NER system and probes for affinity modification experiments.

MATERIALS AND METHODS

The following reagents were used in the work: T4 polynucleotide kinase, T4 DNA ligase, Taq polymerase, deoxynucleoside triphosphates, and Flu-dUTP (Biosan, Novosibirsk); Benzonase Nuclease (Novagen, Germany); λ exonuclease (NEB, USA); proteinase K and ATP (disodium salt) (Sigma, USA); Precision Plus molecular weight protein markers (BioRad, USA); Protease Inhibitor Cocktail Tablets (Roche, Germany); deoxyoligonucleotides (ONT), [γ-^{32}P]ATP (3000 Ci/mmol), and [α-^{32}P]dCTP (3000 Ci/mmol) produced at the Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences (ICBFM SB RAS) (Novosibirsk). Fap-dCTP was synthesized as described in [23] and kindly provided by S. V. Dezhurov (ICBFM SB RAS). DMEM culture medium was produced by Gibco (USA). NER-competent extract of HeLa cells was prepared by the method described in [22]. Recombinant RPA was isolated from *Escherichia coli* strain as described in [24] with certain modifications. Recombinant heterodimer XPC-HR23B (Flag-XPC and in 6His-Tag-HR23B) was obtained by the method described in [23] with slight modifications. Recombinant DNA polymerase β, recombinant PARP1, and DNA marker (pBlueSK/MspI) preparations were kindly provided by S. N. Khodyreva, M. V. Sukhanova, and E. A. Khrapov (ICBFM SB RAS), respectively. The components for polyacrylamide gel preparation and the main components of buffer systems were produced by Sigma or were domestic products with “high-purity” grade.

ONT-1-13 for creation of model DNA duplexes, produced at ICBFM SB RAS, were of the following composition, respectively:

1) 5′-ttaaattgctaacgcagtcagg-3′,

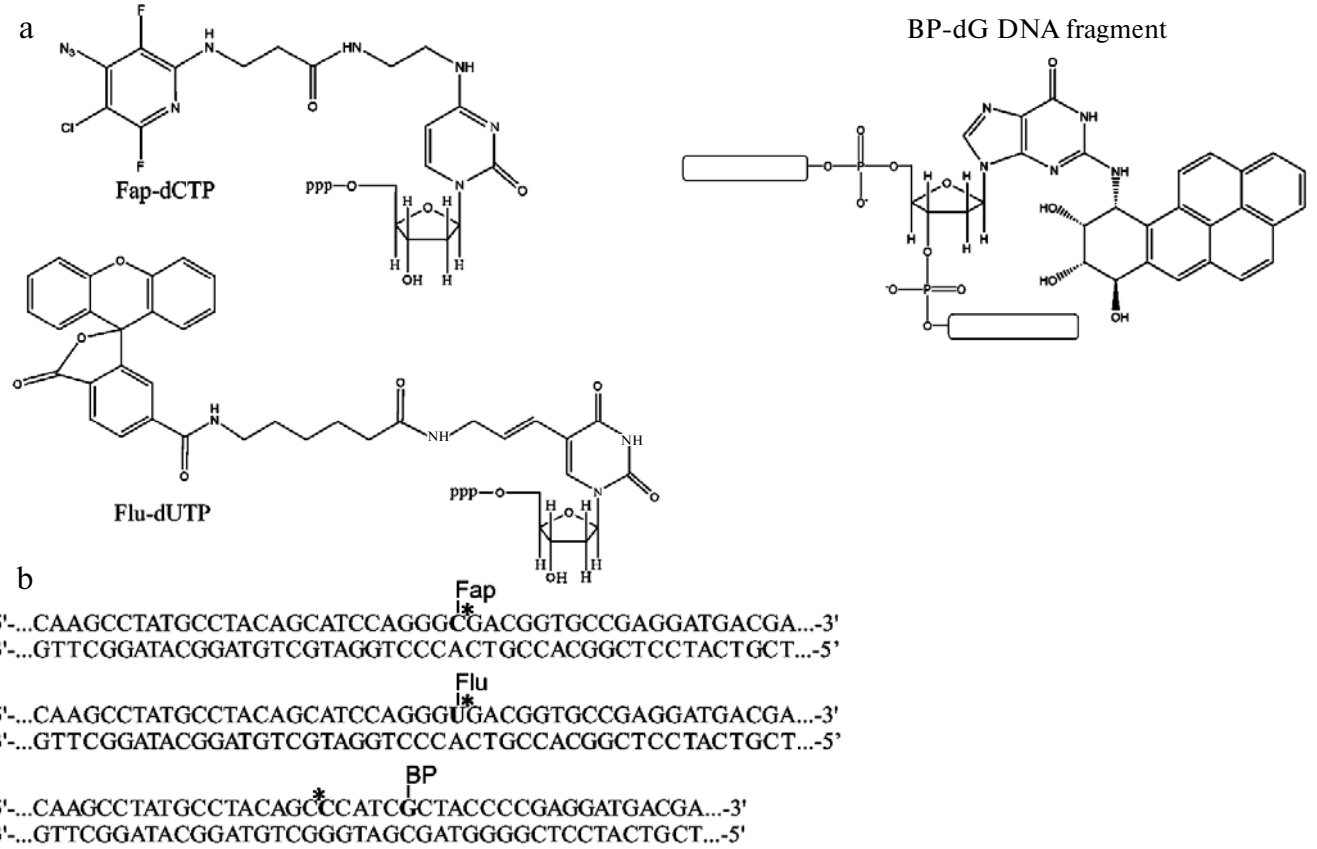

Fig. 1. Model lesions. a) Structures of dCTP and dUTP analogs and the fragment of deoxyoligonucleotide containing (+)-*cis*-benzo[a]pyrene-*N2*-deoxyguanine (BP-dG) used for the synthesis of modified DNA chains. b) Fragments of created double-stranded DNA containing modified nucleotides. * Position of radioactive phosphate.

2) 5′-p-ttaaattgctaacgcagtcagg-3′,

3) 5′-tggacgatatcccgcaagaggc-3′,

4) 5′-tggacgatatcccgcaagaggcccggcagtaccggcataaccaagc-ctatgcctacagcatccaggg-3′,

5) 5′-gacggtgccgaggatgacgatgagcgcattgttagatttcatacacgg-tgcctgactgcgttagcaatt-3′,

6) 5′-tggacgatatcccgcaagaggcccggcagtaccggcataaccaagc-ctatgcctacagc-3′,

7) 5′-p-ccgaggatgacgatgagcgcattgttagatttcatacacggtgcct-gactgcgttagcaat-3′,

8) 5′-cctcggcaccgtcgccctggatgctgtagg-3′,

9) 5′-gggggctcggcaccgtcaccctggatgctgtagg-p-3′,

10) 5′-gtcatcctcggggtagcgatgggctgtag-3′,

11) 5′-aattgctaacgcagtcaggcaccgtgtatgaaatctaacaatgcgctc-atcgtcatcctcggggtag-3′,

12) 5′-p-cgatgggctgtaggcataggcttggttatgccggtactgccgggcc-tcttgcgggatatcgtcc-3′,

13) 5′-ctacagcccatcgctaccccgaggatg-3′,

where “p” in the ONT structure stands for the 5′- or 3′-terminal phosphate. ONT-14 5′-ccatcG*-ctacc-3′, where G* is a BP-modified nucleotide (Fig. 1), has been synthesized as described in [23] and kindly provided by N. E. Geacintov (New York University, USA).

The preparation of a 140-bp duplex was obtained by PCR in a reaction mixture containing: 1× Taq DNA polymerase buffer, 0.25 mM dNTP, 1 mM $MgCl_2$, 0.75 μM primers (ONT-1 and -3, or ONT-2 and -3), 25 ng of a template (DNA pBR322), and 4-6 activity units of Taq polymerase per 100 μl of reaction mixture. The reaction mixture was analyzed in 10% polyacrylamide gel, 1× TBE under nondenaturing conditions. MspI-hydrolysate of pBSK was used as a length marker.

Unmodified single-stranded DNA (140 bp) was obtained by enzymatic reaction catalyzed by λ exonuclease. DNA duplex (10 μM) was used as a substrate; one of its chains was phosphorylated at the 5′-end. The reaction was performed under conditions suggested by the supplier.

Modified dNMP were introduced into the 3′-end of ONT using DNA polymerase β. The 67-bp initiating oligonucleotide (ONT-4) was mixed with the template (ONT-8) at molar ratio 1 : 1 in the buffer for β-polymerase. The test tube with the mixture was placed into a thermostat at 90°C and slowly cooled to room temperature. When completing the DNA primer with dNTP analogs, the reaction mixture contained a template-primer DNA duplex (0.2 μM), a dNTP analog (50 μM), and DNA polymerase β (1-2 μg per 20 μl of reaction mixture). The reaction was carried out at 37°C for 30-60 min and stopped by heating the mixture for 15 min at 65°C.

For ligation, 5′-[^{32}P]ONT-5 (specific radioactivity 300 Ci/mmol) was added in equimolar amount to the reaction mixture containing long ONT-4 (the 3′-OH component of ligation reaction) and template ONT-8. The modified chain not containing a radioactive label was synthesized using the unlabeled 5′-P component of ligation reaction (5′-P-ONT-5) and the initiating primer with low specific radioactivity carrying ^{32}P-label at the 5′-end. The reaction mixture was incubated at 90°C for 10 min; then the mixture was slowly cooled to room temperature for fusion of the duplex. In addition to the DNA duplex with a single-stranded break flanked by modified dNMP on the 3′-side and by phosphoryl residue on the 5′-side, the reaction mixtures for ligation contained DNA ligase T4 (8 activity units/μl) and ATP (1 mM). The reaction was carried out at 16 or 4°C for 2 or 16-18 h, respectively.

For isolation of the modified 137-bp ONT, the reaction mixture components after ligation were separated by electrophoresis in polyacrylamide gel under denaturing conditions. The target ONT was isolated from the gel according to [19]. The amount of modified ONT in the final preparation was assessed using Molecular Imager FX Pro$^+$ and Quantity One software (BioRad).

For the formation of model DNA duplexes, the solution containing complementary modified 137-bp and unmodified 140-bp ONT in stoichiometric ratio 1 : 5, as well as 10 mM Tris-HCl, pH 7.8, and 1 mM EDTA, was put into a thermostat and kept at 90°C for 10 min. After slow (~1°C/min) cooling to room temperature, the resulting DNA duplexes were analyzed by electrophoresis under denaturing conditions using 10% polyacrylamide gel, 1× TBE. In all cases the duplex formed was above 95%.

The BP-modified DNA chain was obtained by ligation of 11-bp 5′-[^{32}P]ONT-14 (specific radioactivity of 3000 Ci/mmol) and flanking ONT-6 (3′-component) and ONT-7 (5′-component) fused in equimolar amounts on template ONT-10. The synthesized DNA chain was 131 nucleotides in length. Isolation of the target modified ONT and formation and purification of the model DNA duplex were also performed by the method described above. Complementary unmodified chain in this case was obtained by ligation of ONT-11 and ONT-12 on template ONT-13.

Photoaffinity modification of proteins using Fap-dC-DNA was performed in reaction mixture (10-30 μl) containing photoreactive DNA and the respective proteins in the buffer for modification (25 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 25 mM KCl, 0.125 mM β-mercaptoethanol). Samples were exposed to UV radiation (312 nm) in a BIO-LINK®BLX (Vilber Lourmat) for 5 min at 3 J/cm^2·min.

DNA–protein adducts were treated with benzonase after completion of the UV radiation. The reaction mixtures with added benzonase (0.1 activity unit/μl of reaction mixture) were incubated for 30 min at 37°C; then the reaction products were analyzed by SDS-PAGE according to Laemmli. If necessary, the proteins were stained in the gel with Coomassie R-250 solution.

Autopoly(ADP-ribosyl)ation of PARP1 was performed in the presence of 1 mM NAD^+ for 30 min at 37°C.

The efficiency of removal of the modified DNA region by the method of direct detection was determined by incubating the reaction mixture (40 μl) containing 1× NER buffer (25 mM Hepes, pH 7.8, 45 mM NaCl, 4.4 mM $MgCl_2$, 0.1 mM EDTA, 4 mM ATP), 5 nM of radioactive modified duplex, and NER-competent cell extract for 15-40 min at 30°C. The reaction was stopped by adding 0.5 μl of proteinase K solution (5 μg/ml), and the mixture was kept for 40 min at 30°C. For deproteinization, an equal volume of phenol–chloroform–isoamyl alcohol mixture (25 : 24 : 1) was added to the reaction mixture and centrifuged (2 min, 13,400*g*). The collected water phase was washed once with chloroform–isoamyl alcohol mixture (24 : 1). The DNA was precipitated by ethanol in the presence of NaCl. Reaction products were analyzed by electrophoresis in polyacrylamide gel under denaturing conditions followed by quantitative radioautography.

The efficiency of removal of nonradioactive DNA region containing a modification was determined using [α-^{32}P]dCTP and the DNA polymerase reaction. The reaction mixture containing 1× NER buffer, 5 nM of modified duplex, and 7 μl of NER-competent cell extract per 40 μl of reaction mixture was incubated at 30°C (10-40 min). Then the template (ONT-9) was added to the reaction mixture to 500 nM and 4 μl of 10× buffer of Taq DNA polymerase. The mixture was heated to 95°C and then kept for 10 min at room temperature. After adding Taq polymerase (15 activity units) and [α-^{32}P]dCTP (3-5 μCi), the mixture was incubated for 5 min at 40°C followed by addition of 1.5 μl of solution containing dATP, dGTP, dTTP (100 μM), and dCTP (50 μM) and then incubated again for 15 min. Deproteinization and sample analysis were carried out as described above.

The effect of Fap-dC- and Flu-dU-DNA on the level of specific excision from [^{32}P]BP-dG-DNA was studied. The reaction mixtures containing 5 nM [^{32}P]BP-dG-DNA and 1× NER-buffer were incubated for 40 min

at 30°C in the absence or presence of 5 nM unlabeled competitive Fap-dC- or Flu-dU-DNA and 15 μg/μl of the HeLa extract proteins. Deproteinization and sample analysis were carried out as described above.

RESULTS AND DISCUSSION

Using the improved enzymatic method of synthesis, we have created long Fap-dC- and Flu-dU-DNA (137 bp) and analyzed their properties as substrates for the excision reaction catalyzed by the proteins of NER-competent extract of HeLa cells. BP-dG-DNA (131 bp) synthesized with the respective modified ONT was used as a standard substrate. The structural formulas of model lesions used in the work and schematic representation of the DNA fragments containing modifications are shown in Fig. 1.

The activity demonstrated by NER at the stage of lesion removal is measured by two methods: direct and indirect detection of excision products. During the direct detection, Fap-dC-DNA and Flu-dU-DNA contained a ^{32}P-label immediately in the damaged nucleotide (on the 3′-side); in the case of BP-DNA, the label was located at a distance of five nucleotides on the 5′-side of the lesion. Thus, excision products (22-32-nucleotide DNA fragments bearing lesions in a position somewhat farther from the 5′-end) must contain also the ^{32}P-label. The advantage of this method is the possibility to quantitatively assess lesion removal efficiency without additional stages.

DNA modifications induced by benzo[a]pyrene in humans are one of the most widespread and well-studied types of lesions repaired by NER [1-3, 22, 23]. [^{32}P]BP-DNA was synthesized using modified ONT-14 containing a (+)-*cis*-stereoisomer of benzo[a]pyrene-*N2*-deoxyguanidine flanked on the 5′- and 3′-sides by dCMP residues. This model lesion belongs to a class of systematically studied and rather effective synthetic NER substrates. However, it should be noted that the level of excision in experiments *in vitro* rarely exceeds 5% even for such model DNA [21, 22]. During the storage of [^{32}P]BP-DNA with the proteins of NER-competent HeLa extract, radioactive DNA fragments of 22-32 nucleotides were observed in the mixture. The quantity of specific hydrolysis products increased during 10-60 min from the beginning of the reaction and under increase in the concentration of extract proteins in the reaction mixture within the analyzed range of concentrations (2-15 μg/μl) (data not shown). Figure 2a presents the results of reaction mixture analysis after 40 min of incubation of [^{32}P]BP-DNA with the extract proteins. The level of excision of damaged fragments from [^{32}P]Fap-dC- and [^{32}P]Flu-dU-DNA was lower than the level detected by such method.

Excision of fragments of typical length from Flu-dU-DNA was registered when using the method of indirect detection of excision products (Fig. 2b). Radioactive

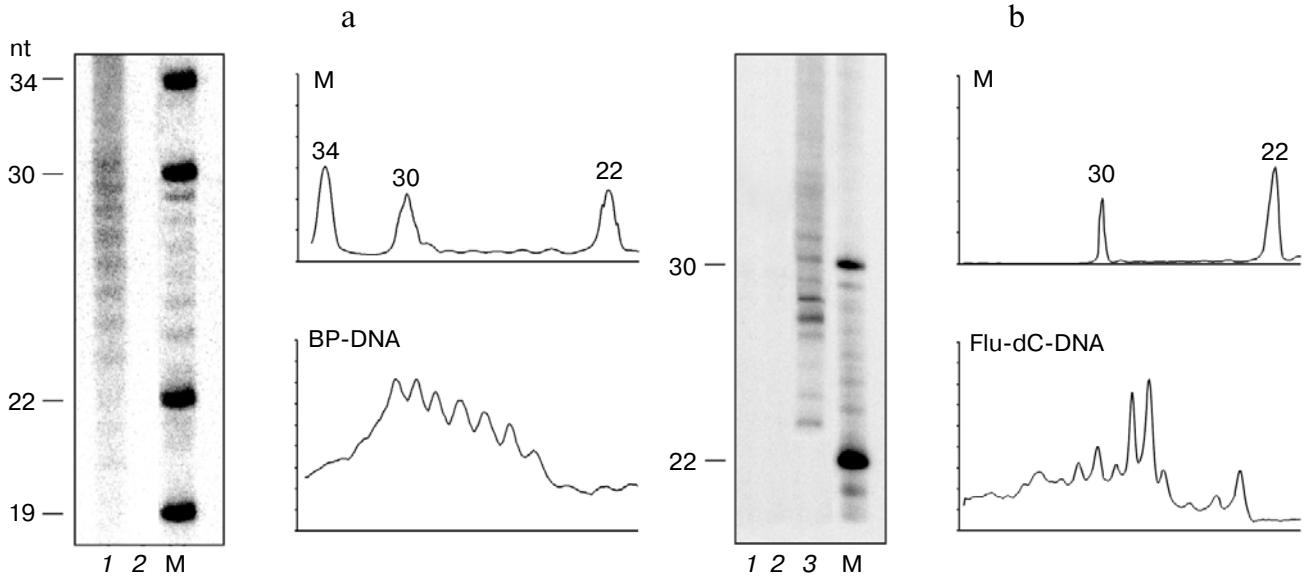

Fig. 2. Radioautographs of gels after separation of excision reaction products. a) Direct detection of excision products. *1*) Analysis of reaction mixture containing 5 nM [^{32}P]BP-DNA and 15 μg/μl of the HeLa extract proteins in 1× NER buffer (25 mM Hepes, pH 7.8, 45 mM NaCl, 4.4 mM $MgCl_2$, 0.1 mM EDTA, 4 mM ATP) incubated for 40 min at 30°C; *2*) modified duplex incubated without the extract; M, here and in Fig. 3, markers of DNA length (nt, nucleotide). b) Indirect detection of excision products. Reaction mixtures contained nonradioactive Fap-dC-DNA or Flu-dU-DNA at 5 nM and 15 μg/μl of the HeLa extract proteins. Excision products fused with the respective template were completed using Taq polymerase with [α-^{32}P]dCTP as a substrate. *1*) Unmodified DNA; *2*) Fap-dC-DNA; *3*) Flu-dU-DNA. On the right, (a) and (b) panels show the results of densitometric analysis of the radioautographs. In both cases the products were separated by electrophoresis under denaturing conditions (10% polyacrylamide gel, 7 M urea).

α-[^{32}P]dCMP was introduced as a label into the removed DNA fragments with damaged nucleotides using DNA-polymerase reaction and complementary template ONT. The advantages of such estimation of substrate properties are higher sensitivity and absence of necessary synthesis of model DNA with high specific radioactivity. This approach, however, prevents assessment of the number of damaged DNA molecules processed by NER proteins. Besides, it is obvious that the length of detected radioactive products does not correspond to the true length of cut-out fragments of the damaged chain, and the pattern of excision products on the whole may be different from the initial one. However, products of specific excision from Fap-dC-DNA were not revealed by this method either.

Previously, an analogous method was used for estimation of the activity shown by the excision complex of NER-competent extracts towards Fap-dU (-dC) introduced into ring model substrates [17]. The values for Fap-dU- and Fap-dC-DNA were, respectively, no more than 20 and ~8% of the activity shown towards AAF-dG-containing DNA substrate used as a standard. The level of specific excision of damaged fragments from the linear DNA may additionally decrease due to the binding of Ku-antigen present in HeLa extracts to such substrates [25].

Flu-dU- and Fap-dC-DNA demonstrated the ability for effective competition with BP-DNA for the interaction with proteins of the cell extract excision complex. The experiments (results are shown in Fig. 3) were performed using [^{32}P]BP-DNA and nonradioactive competitive DNA duplexes. In the presence of an equimolar amount of the control unmodified duplex, the level of excision for [^{32}P]BP-DNA was no less than 95% of the level of excision observed in the absence of competitive DNA. As a result of adding the equimolar amount of Flu-dU- or Fap-dC-DNA to the reaction mixture, the level of excision of damaged fragments from [^{32}P]BP-DNA significantly decreased. In the presence of Fap-dC-DNA, the level of excision from BP-DNA was lower by 20%, while the presence of Flu-dU-DNA reduced the level of BP-DNA specific hydrolysis twofold. Decrease in the quantity of radioactive cleavage products is evidently due to distribution of NER proteins between radioactive BP-DNA and nonradioactive Fap-dC- or Flu-dU-DNA.

Our results, in combination with the data of previous studies [14, 16, 19], allow us to consider the linear 137-bp

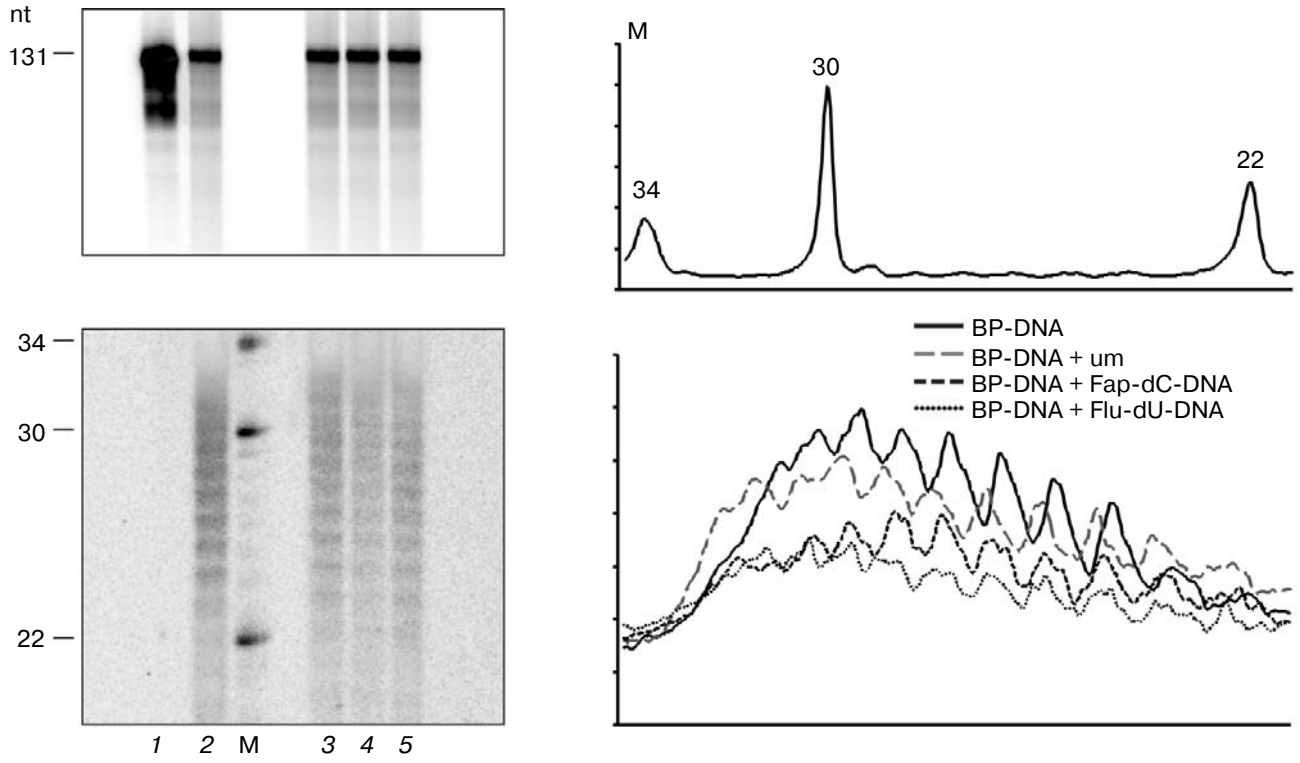

Fig. 3. Radioautograph of gel after separation of excision reaction products by electrophoresis under denaturing conditions (10% polyacrylamide gel, 7 M urea). Reaction mixtures containing 5 nM [^{32}P]BP-DNA and 1× NER buffer were incubated for 40 min at 30°C in the absence (*2*) or presence of 5 nM unlabeled competitive DNA ((*3*) unmodified (um) duplex, (*4*) Flu-dU-DNA, (*5*) Fap-dC-DNA) and 15 μg/μl of the HeLa extract proteins. *1*) Results of separation of the mixture containing only unmodified duplex stored with the extract proteins. The products were separated by electrophoresis under denaturing conditions (10% polyacrylamide gel, 7 M urea). The result of densitometric analysis of the radioautograph is shown on the right; the lengths of marker DNA are given on the left.

Fap-dC-DNA as unrepairable NER substrates. Unrepairable substrate analogs, i.e. structures capable of selective binding of DNA repair proteins but not liable to further processing, are most dangerous for cells. Accumulation of such DNA can considerably reduce the efficiency of the repair machinery due to immobilization of its protein components within nonproductive nucleoprotein complexes [26]. However, the ability of model lesion to effectively inhibit the repair process *in vitro* indicates the possibility of its application as a prototype for creation of selective DNA repair inhibitors contributing to the enhancement of efficiency in chemotherapy. It is also evident that photoactive unrepairable analogs of NER lesions are of great interest as elements of architecture of affinity DNA probes.

Photoaffinity modification is quite successively used for studying the dynamics of functioning of protein complexes and in modern proteomics [27-29]. Covalent fixation of proteins interacting with the damaged DNA region becomes possible in the presence of photoactivated lesion in a substrate analog. Affinity modification coupled with the methods based on application of specific antibodies, gel retardation, and the equilibrium methods of quantitative assessment of protein affinity to DNA made it possible, in particular, to elucidate some details of the mechanism of the preincision stage of GG-NER *in vitro* and the role and mutual influence of protein factors involved in this process [6, 7, 13-19].

We have used Fap-dC-DNA (137 bp) containing a ^{32}P-label in the photoactive nucleotide residue for revealing the proteins of NER-competent extract of HeLa cells specifically interacting with model DNA of such type. Figure 4 presents the results of electrophoretic analysis of photocrosslinked products. Cell extract contains a wide range of DNA-binding proteins; however, UV radiation of the reaction mixture containing Fap-dC-DNA and HeLa cell extract results in formation of a limited number of major covalent DNA–protein adducts, which is evi-

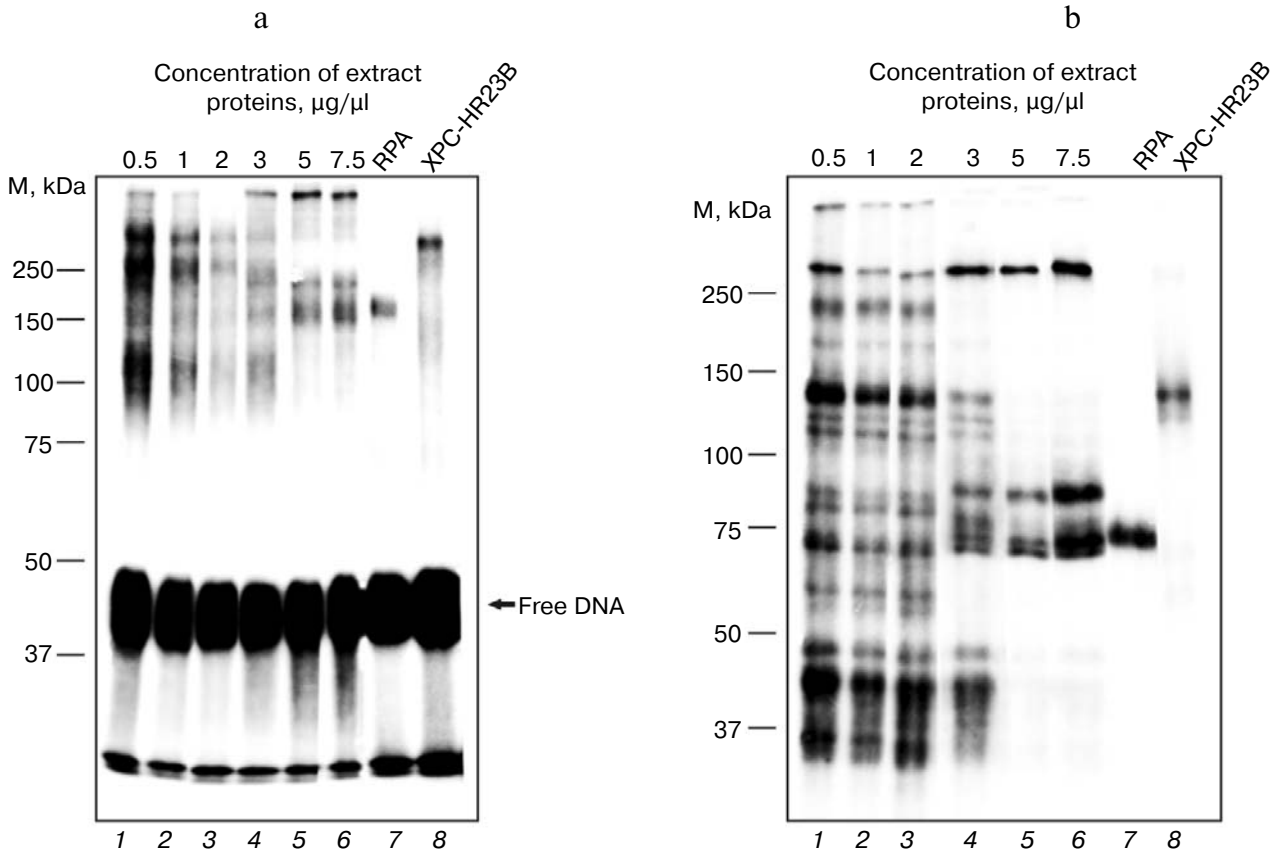

Fig. 4. Radioautographs of gels after separation of products of photolinking of proteins to DNA. Reaction mixtures contained 10 nM [^{32}P]Fap-dC-DNA, 0.75 to 7.5 μg/μl of the HeLa extract proteins (*1-6*), or recombinant proteins: 1.7 μM RPA (*7*) and 0.5 μM XPC-HR23B (*8*), as well as 25 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 25 mM NaCl, 0.125 mM β-mercaptoethanol. The mixtures were exposed to UV radiation at 312 nm and intensity of 3 J/cm^2·min for 5 min. After the UV exposure, aliquots taken from each mixture were treated with benzonase preparation (1 activity unit per 10 μl of a sample, 30 min at 37°C). The reaction was stopped by heating with the denaturing buffer for protein samples. The buffer for samples was also added to the residual part of the reaction mixture. a) Analysis of samples not treated with benzonase; b) analysis of samples treated with benzonase. Products of photolinking were separated by electrophoresis in 10% polyacrylamide gel according to Laemmli. M, molecular weights of marker proteins.

dence of selectivity of interaction. The efficiency of this interaction is high: up to 25% of photoactive DNA substrate was spent for the formation of covalent adducts with extract proteins. The high selectivity and efficiency of Fap-dC-DNA as affinity probes were also observed with the shorter (48-bp) DNA duplexes for modification of extract proteins [19]. However, photolinking with the long Fap-dC-DNA (137 bp) revealed a number of quantitative and qualitative differences in modification patterns. The experiments with 137-bp Fap-dC-DNA showed a distinct dependence of the spectrum of major modification target proteins and the levels of their labeling on the concentration of extract proteins in the mixture. Decrease in the concentration of extract proteins resulted in extension of the set of formed radioactive nucleoprotein adducts (Fig. 4a, lanes *1* and *6*).

It is obvious that covalent linking of the long DNA probe significantly changes the electrophoretic mobility of the modified proteins. Besides, it is known that modification of the same peptide may result in appearance of products with different electrophoretic mobility [19]. In the presence of ^{32}P-label within a photoactive lesion, just as in case of using 48-bp Fap-dC-DNA [19], it was possible to expose the reaction mixtures after photolinking to benzonase treatment for more adequate estimation of molecular weights of target polypeptides. Benzonase, being a nonspecific endonuclease, cleaves the DNA within the nucleoprotein adduct, with the exception of a few nucleotides located immediately near the place of covalent linking of DNA to protein. The target proteins of modification remained ^{32}P-labeled after such treatment as they contained a label in the photoactive lesion, and their electrophoretic mobility became equal or quite close to the mobility of the respective unmodified polypeptides. This is well illustrated by comparison of the results of analysis of reaction mixtures after modification of purified recombinant proteins (RPA and XPC-HR23B in Fig. 4b). The character of dependence of the modification pattern on extract protein concentrations in reaction mixtures after the treatment of reaction products with benzonase was invariable on the whole (Fig. 4b). However, opposite directions of the effects of total concentration of extract proteins on the level of labeling of some target polypeptides were clearly demonstrated. For instance, the decrease in total protein concentration was accompanied by decrease in the level of modification of one of the two ~70-kDa polypeptides and the ~88-kDa protein, while the level of modification of the ~127-kDa polypeptide abruptly increased. For the 48-bp Fap-dC-containing duplexes such marked changes in modification patterns as a result of variations in extract protein concentrations were not shown [19]. The set of main targets of labeling with 137-bp DNA included polypeptides of the extract with the same values of apparent molecular weights (35-90 kDa) as when using the shorter (48 bp) Fap-dC-DNA.

A considerable part of modification products of the ~70- and ~88-kDa polypeptides may be formed by subunits of the Ku-antigen included in the complex of DNA-dependent protein kinase (DNA-PK). At high concentrations of extract proteins, these polypeptides are the main targets of intensive labeling for both short and long Fap-dC-DNA. Heterodimer Ku70/80 characterized by high affinity to double-stranded DNA ends and the ability for subsequent translocation along the molecule [25] may be found in immediate proximity to the photoactive chain fragment located in the internal position of DNA. As noted in work [30], the Ku-antigen in HeLa cells represented by a great number of copies and is present in high concentrations in the corresponding cell extracts. This has been demonstrated by the highly efficient linking to chemically active DNA bearing the AP-site [31]. Ku70/80 and DNA-PK were found among the major targets of modification and during interaction with the proteins of HeLa cell extracts of NER substrate analogs such as DNA of different length and structure containing platinum adducts and a benzophenone derivative on their basis [29]. Thus, Ku70/80 labeling may be rather intensive even in the absence of high specificity to the DNA probes used.

Previously it has been shown that RPA with high affinity interacts with Fap-dC-DNA (48 bp) by contacting the damaged region of the duplex via the large (70 kDa) subunit [14], which proves to be a target of modification in NER-competent HeLa [19]. This fact suggests that the second band of labeled polypeptide with a weight close to 70 kDa, which is present in the labeling pattern in a broad range of extract protein concentrations, is the large RPA subunit.

As mentioned above, the long DNA probe, in contrast to the previously used 48-bp duplex, is capable of efficient and specific interaction with extract polypeptides of more than 100 kDa. This interaction was especially effective at concentrations of the extract proteins <3 μg/μl (Fig. 4b).

Specifically, analysis of benzonase-treated reaction mixtures has shown that the long Fap-dC-DNA intensively forms a covalent adduct with a protein of apparent molecular weight close to 127 kDa at extract protein concentration of 0.5-3 μg/μl (Fig. 4b). The level of formation of this covalent adduct is considerably reduced on further increase in protein concentration. Comparison of modification patterns of the extract proteins and the purified recombinant XPC-HR23B demonstrates coincidence of electrophoretic mobilities of the 127-kDa target protein and the XPC subunit of the heterodimer. However, it should be taken into account that the contribution to formation of labeling products with the same electrophoretic mobility in a multicomponent system such as cell extract can be made by several polypeptides of similar molecular weight capable of specific interaction with the DNA used. In particular, the DNA-binding subunit of factor XPC and the large subunit of a five-subunit factor

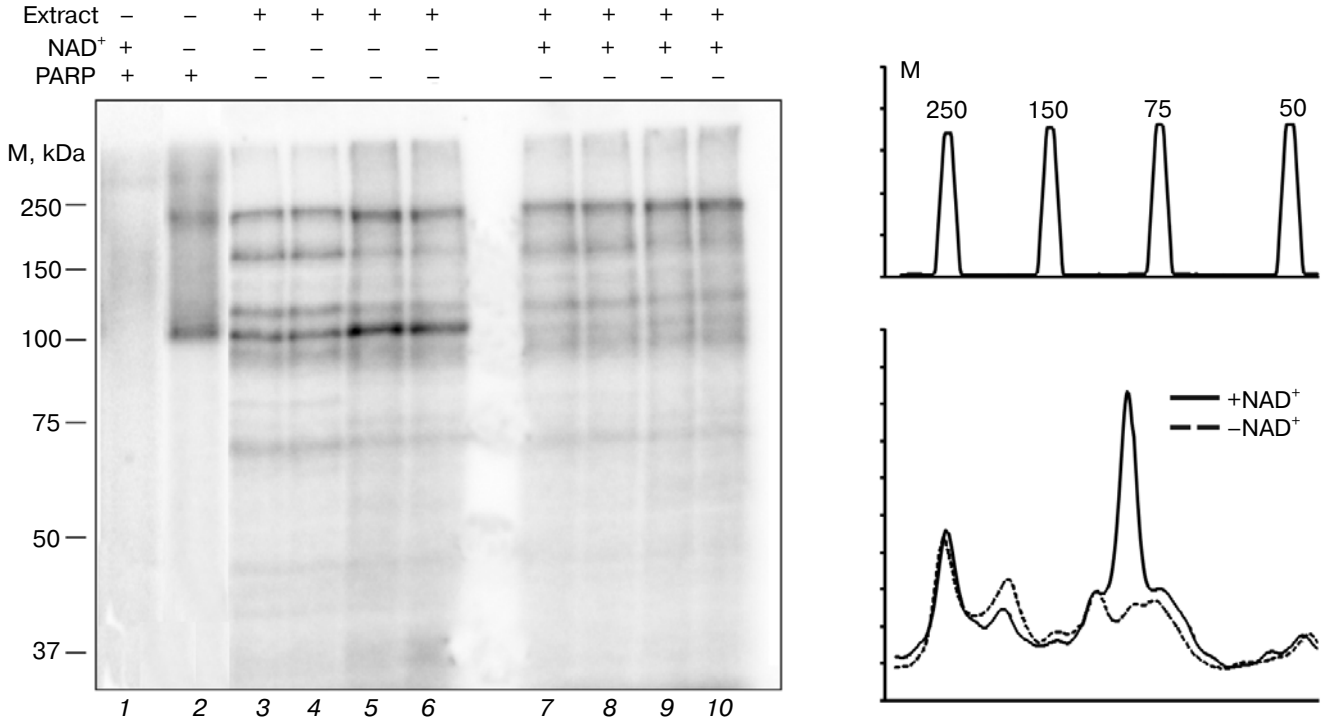

Fig. 5. Radioautographs of gels after separation of products of photolinking of proteins to DNA. Reaction mixtures for modification contained 10 nM [^{32}P]Fap-dC-DNA and 7.5 μg/μl (*3*, *4*, *7*, *8*) or 4 μg/μl (*5*, *6*, *9*, *10*) of the HeLa extract proteins, or recombinant protein PARP1 at 0.1 μM (*1*, *2*), and 1 mM NAD^+ (*1*, *7-10*). The reaction was carried out in buffer containing 25 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 25 mM NaCl, 0.125 mM β-mercaptoethanol. The mixtures were exposed to UV radiation at 312 nm and intensity of 3 J/cm^2·min for 5 min; the samples were treated with benzonase (1 activity unit per 10 μl of the sample). The reaction was carried out for 30 min at 37°C and stopped by heating with denaturing buffer for protein samples. The products of photolinking were separated by electrophoresis in 10% polyacrylamide gel according to Laemmli. M, molecular weights of marker proteins. The result of densitometric analysis of the radioautograph is shown on the right.

RFC (RFC1) included in the set of core NER proteins are nearly of the same molecular weight [1-3]. RFC1 and minor subunits of this factor with molecular weights of 35 to 40 kDa were found among proteins of the HeLa extract forming cross-links with Pt-DNA [29]. For identification of polypeptides forming covalent adducts, it is necessary to use methods based on application of specific antibodies, mass spectrometry, or functional tests.

The products of modification of the ~115-kDa protein were formed in the same range of extract protein concentrations with almost equal intensity (Fig. 4b). The appropriate molecular weight and rather high content in HeLa cells are typical, in particular, of poly-(ADP-ribose)-polymerase-1 (PARP1, 112 kDa). The protein corresponding to PARP1 in mobility was present in the modification pattern when using short (48 bp) Fap-dC-containing duplexes; however, the relative level of its linking was much lower [19]. Recently it has been shown that PARP1 initially defined as a sensor of single-stranded breaks in DNA and intensively studied as a regulator protein of the pathways of base excision repair and programmed cell death [32-34] can specifically interact with another group of NER substrates: DNA treated with platinum-based preparations [29, 35]. It is known that PARP1 can be autopoly(ADP-ribosyl)ated in the presence of NAD^+. Automodification alters many properties of this protein, including the affinity to DNA and electrophoretic mobility. The ability for autopoly(ADP-ribosyl)ation is maintained in PARP1 even after covalent linking to DNA. However, in this case the type of modification pattern is changed: the band corresponding to PARP1 linking products disappears [33, 34]. We have used this functional test to identify the polypeptide with molecular weight of ~115 kDa.

The storage of modification products with NAD^+ resulted in complete disappearance of the band of modified recombinant PARP1 and abrupt decrease in intensity of the respective band in the labeling pattern of extract proteins (Fig. 5). Incubation of photolinked products under the same conditions in the absence of NAD^+ caused no changes in modification pattern. Consequently, the 115-kDa protein of HeLa cell extract effectively forming covalent adducts with Fap-dC-DNA can be identified as PARP1.

Thus, analysis of the interaction between the long Fap-dC-DNA and the proteins of cell extract containing

a functionally active NER protein complex indicates the given model DNA is an unrepairable substrate that is able to compete for the binding of NER enzymes and factors and is an effective probe for the study of protein complexes that catalyze nucleotide excision repair, as indicated by photoaffinity modification.

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