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A New Binary System for Photosensitized Labeling of DNA Polymerases in Nuclear Extract

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Abstract—A binary system of reagents was used for photosensitized labeling of proteins of bovine testis nuclear extract. A dUTP analog containing 4-azido-2,5-difluoro-3-chloropyridyl group (FAP-dUTP) was used for the first time as a component of the binary system, and a dUTP analog containing the pyrenyl group (Pyr-dUTP) was used as a photosensitizer. Photoaffinity labeling of proteins of nuclear extract was performed using the radioactively labeled DNA duplex with the photoreactive FAP group at the 3′-end of elongating DNA strand and analog of the deoxyribose phosphate residue (3-hydroxy-2-hydroxymethyltetrahydrofuran (F) 5′-phosphate) at the 5′-end of the nick. Such structure is formed by the action of nuclear extract enzymes from the initial DNA duplex containing a synthetic apurine/apyrimidine site and is a photoreactive analog of a long-patch base excision repair intermediate. UV-irradiation modified a limited number of proteins of the nuclear extract. As shown using specific antibodies, the new binary system of photoreagents increases the efficiency of DNA polymerase β labeling.

Key words: photoaffinity labeling, photoreactive dNTP analogs, binary system of photoaffinity reagents, bovine testis nuclear extract

Photoaffinity labeling by base-substituted dNTP analogs is widely used for study of the structure and functions of DNA polymerases and other DNA-binding proteins [1]. dNTP analogs containing photoactivated groups attached to the exocyclic amino group of cytosine and the 5-position of uracil appeared to be efficient substrates for all investigated DNA polymerases—HIV reserve transcriptase [2, 3], thermostable DNA polymerase *Tte* [4], DNA polymerase α-primase [5, 6], and DNA polymerase β [5]. Kinetic characteristics of dNTP analogs (K_m and V_{max}) are close to those of the natural dNTPs. This property of photoreactive dNTPs allows their use as substrates for DNA polymerases to obtain the photoreactive DNA primers and their subsequent use for affinity labeling of DNA polymerases and factors of DNA replication and repair [2-6].

Labeling efficiency, i.e., the level of formation of covalent reagent–biopolymer adducts, is an important characteristic of affinity reagents. Earlier it was mentioned that variation of the photoreactive group type in the composition of a reagent influences the efficiency of photoaffinity labeling of DNA polymerases by photoreactive DNA primers; reactive groups which were crosslinked with the maximal efficiency with the protein and DNA targets were revealed [7]. Further increase in efficiency of protein labeling via creation of new photoreactive groups is needed for production of sufficient amounts of the labeled proteins for their subsequent identification, for example, using mass spectrometry. Synthesis and use of new reagents for increasing the efficiency of protein labeling in multicomponent systems such as cell and nuclear extracts are of particular interest for subsequent identification of proteins interacting with photoreactive intermediates of DNA replication and repair. Recently photoaffini-

Abbreviations: FAB-dUTP) 5-[N-(2,3,5,6-tetrafluoro-4-azidobenzoyl)-3-amino-*trans*-propen-1-yl]-2′-deoxyuridine-5′-triphosphate; FAP-dUTP) 5-{N-[N-(4-azido-2,5-difluoro-3-chloropyridine-6-yl)-3-aminopropionyl]-*trans*-3-amino-propen-1-yl}-2′-deoxyuridine-5′-triphosphate; Pyr-dUTP) 5-[N-(4-(1-pyrenyl)-ethylcarbonyl)-3-amino-*trans*-propen-1-yl]-2′-deoxyuridine-5′-triphosphate; BER) base excision repair; FEN-1) flap-endonuclease 1; APE) apurinic/apyrimidinic endonuclease; PARP-1) polyadenylriboso-polymerase 1.

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ty labeling was successfully applied for identifying proteins interacting with photoreactive intermediates of base excision repair in mouse fibroblast cell extract [8].

To increase selectivity of photoaffinity labeling of catalytic subunits of DNA polymerases in the presence of other DNA- and dNTP-binding proteins, a method using a binary system of photoaffinity reagents—photoreagents and sensitizers—was developed [9, 10]. In this work we tried to increase efficiency of sensitized labeling using dNTP analog containing 4-azido-2,5-difluoro-3-chloropyridyl group (FAP-dUTP) as a photoreagent and dNTP analog containing the pyrenyl group (Pyr-dUTP) as a sensitizer.

A binary system of photoaffinity reagents was used for identification of DNA polymerases participating in base excision repair (BER), which is one of the basic mechanisms for removing damages caused by external and internal agents. So far two routes of BER differing in the number of resynthesized nucleotides are known: short-patch excision repair when only one base is resynthesized, and long-patch excision repair when from 2 to 6 bases are resynthesized. In the case of short-patch repair DNA synthesis is probably performed by DNA polymerase β, whereas the second type of repair can be also performed by other DNA polymerases, for example, δ and/or ε [11]. Recently it was reported that repair of gaps several nucleotides in length depends on DNA polymerase β [12]. Newly discovered polymerases, for example, DNA polymerase ι [13] or λ [14], can also participate in this process. Thus, the question what cell DNA polymerases are responsible for certain repair mechanisms is open. In this study we used photoreactive DNA intermediates of long-patch base excision repair synthesized *in situ* in bovine testis nuclear extract using a photoreactive dUTP analog (FAP-dUTP) as the substrate. Subsequent irradiation in the presence or in the absence of a sensitizer (Pyr-dUTP) allowed identification of DNA polymerase β as the main product detected with the binary system of photoaffinity reagents.

MATERIALS AND METHODS

The following reagents were used in this study: Rainbow molecular mass markers and [γ-^{32}P]ATP from Amersham (USA); oligonucleotides from Genset (France); T_4 polynucleotide kinase from New England Biolabs (USA), reagents for electrophoresis and main components of the buffers from Sigma (USA), and 1-pyrenebutyric acid from Aldrich (USA). Other reagents were of special purity and chemical grade and of Russian production. Synthesis and photochemical properties of dTTP analogs—Pyr-dUTP, FAP-dUTP, and FAB-dUTP—were described earlier [9, 15, 16].

Bovine testis nuclear extract was prepared as described in [17]. Recombinant DNA polymerase β, FEN-1, APE, and PARP-1 were isolated according to standard procedures [18-21].

Radioactive ^{32}P label was incorporated into the 5′-end of oligonucleotide as described earlier [22]. The labeled oligonucleotides were purified using Microspin™ G-25 columns from Amersham Pharmacia Biotech (USA). Complementary oligonucleotides were hybridized for 3 min at 90°C with subsequent slow cooling of the reaction mixture to room temperature.

Radioactively labeled primer was elongated with DNA polymerases of nuclear extract in 10 μl of reaction mixture containing 50 mM Tris-HCl, pH 7.8 (25°C), 50 mM KCl, 10 mM $MgCl_2$, 4 μl of nuclear extract with protein concentration 5-6 mg/ml, 0.2 μM 5′-^{32}P-labeled oligonucleotide duplex, and 10 μM of one of the dUTP analogs—FAB-dUTP or FAP-dUTP. After incubation for 30 min at 25°C, Pyr-dUTP was added to the reaction mixtures, and the latter were additionally incubated for 30 min at 25°C. The reaction was stopped by addition of 5 μl of 0.1% Bromophenol Blue and 50 mM EDTA in 90% formamide. The samples were heated for 5 min at 90°C. The reaction products were separated by electrophoresis in 20% polyacrylamide gel (acrylamide/bisacrylamide, 20 : 1) in the presence of 7 M urea. Electrophoresis was performed in 100 mM Tris-borate buffer, pH 8.3.

Photoaffinity labeling of proteins of nuclear extract was performed in 10 μl of reaction mixture containing 0.4 μM of 5′-^{32}P-labeled oligonucleotide duplex, 10 μM FAP-dUTP or FAB-dUTP, 4 μl of nuclear extract with protein concentration 5-6 mg/ml, and the standard components described earlier. After complete elongation of the primer (30 min, 25°C), 10 μM Pyr-dUTP was added to the reaction mixture. The reaction mixtures were irradiated for 10 min (λ = 365 nm, sensitized modification) or 20 min (λ = 320 nm, "direct" modification). Controls did not contain Pyr-dUTP. UV-irradiation was performed using an HBO W high-pressure mercury lamp with a monochromator from Bausch and Lomb (USA). The products of modification were analyzed by electrophoresis according to Laemmli [23] with subsequent autoradiography. Efficiency of primer elongation with photoreactive dUTP analog and the level of photoaffinity labeling of DNA polymerase β was quantitatively evaluated using a Storm phosphorus-imager from Molecular Dynamics (USA).

RESULTS AND DISCUSSION

The structures of dUTP photoreactive analogs used in this study are presented in Fig. 1a. Photoreactive dUTP analog containing 4-azido-2,5-difluoro-3-chloropyridyl group (FAP-dUTP) was for the first time used as a photoreagent in experiments on sensitized labeling. Earlier it was shown that the FAP group in the composition of

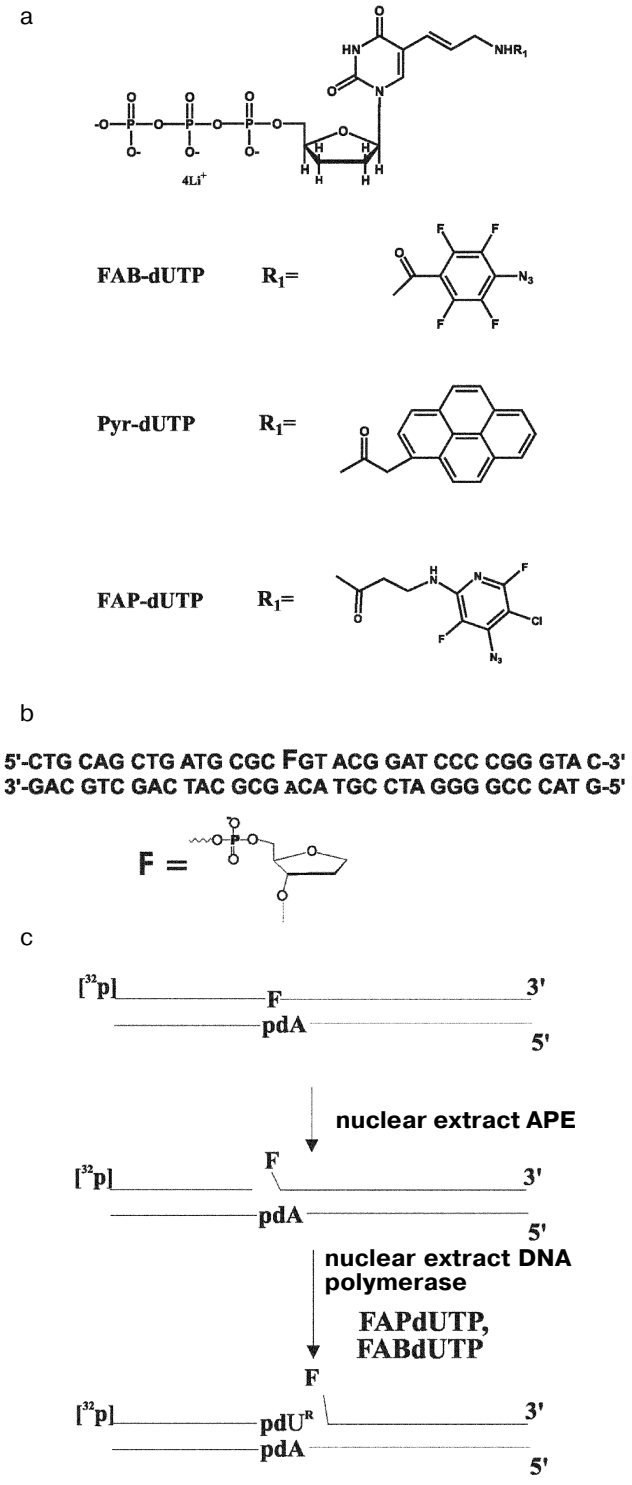

Fig. 1. Structures of photoreactive dUTP analogs and DNA used in this study. a) dUTP analogs containing photoreactive groups: 4-azido-2,5-difluoro-3-chloropyridyl (FAP-dUTP), 2,3,5,6-tetrafluoro-4-azidobenzoyl (FAB-dUTP), or pyrenyl (Pyr-dUTP); b) DNA duplex (34-mer) containing synthetic AP site (3-hydroxy-2-hydroxymethyltetrahydrofuran, **F**) in one strand; c) scheme of synthesis of photoreactive DNA.

DNA modifies DNA polymerases more efficiently than other studied photoreactive groups [7]. Increase in electron-acceptor properties of the aromatic ring due to the presence of the endocyclic nitrogen atom probably causes increase in the level of covalent binding of reagent in the case of FAP-dUTP. High efficiency of reagents containing the same group was also noted in the case of modification of human replicative protein A with reactive oligonucleotides containing the FAP-dUMP residue [24].

Synthesis of photoreactive BER intermediates. A 34-bp oligonucleotide duplex (Fig. 1b) containing in the 5′-^{32}P-labeled strand synthetic AP site, 3-hydroxy-2-hydroxymethyltetrahydrofuran (F), in the position complementary to adenine was used for construction of photoreactive DNA *in situ*. This duplex is a suitable model for study of repair [25, 26], since the F residue modeling the AP site is not excised with lyase activity of DNA polymerase β [27, 28]. In nuclear extract this DNA structure is cleaved by AP endonuclease (APE) with subsequent incorporation of the FAP-dUMP residue into the 3′-end of elongating DNA strand by DNA polymerases using FAP-dUTP as the substrate. As a result, there is formed a photoreactive intermediate of DNA excision repair bearing an analog of deoxyribose phosphate residue (3-hydroxy-2-hydroxymethyltetrahydrofuran-5′-phosphate) at the 5′-end of the nick. The scheme of synthesis of photoreactive DNA is presented in Fig. 1c. Incubation of radioactively labeled DNA in nuclear extract in the presence of FAP-dUTP resulted in incorporation of the only photoreactive dUMP residue into the 3′-end of the nick. The data are presented in Fig. 2. It is interesting that addition of Pyr-dUTP in nuclear extract instead of FAP-dUTP did not result in DNA elongation (Fig. 2, lane *2*), although earlier it was shown that Pyr-dUTP is a good substrate for DNA polymerases including DNA polymerase β [9]. Addition of Pyr-dUTP to nuclear extract after incorporation of FAP-dUMP into the 3′-end of the nick did not result in further elongation of the primer (lane *4*). Consequently, subsequent photoaffinity labeling of proteins of nuclear extract was performed by photoreactive repair intermediates with one dUMP residue containing 4-azido-2,5-difluoro-3-chloropyridyl group at the 3′-end of the nick (lane *3*).

Photoaffinity labeling of nuclear extract proteins. As demonstrated by staining of gel with Coomassie Brilliant Blue after separation of nuclear extract proteins by SDS-PAGE, a large number of proteins are present in nuclear extract (Fig. 3). However, activation of the FAP group (Fig. 4a) as well as the FAB group (Fig. 4b) in the composition of DNA repair intermediate by UV-irradiation with $\lambda = 320$ nm modified a limited number of nuclear extract proteins (Fig. 4, a and b, lane *2*). This result indicates specificity of interaction of nuclear extract proteins and BER intermediates. We used a binary system of photoaffinity reagents for identification of DNA polymerases

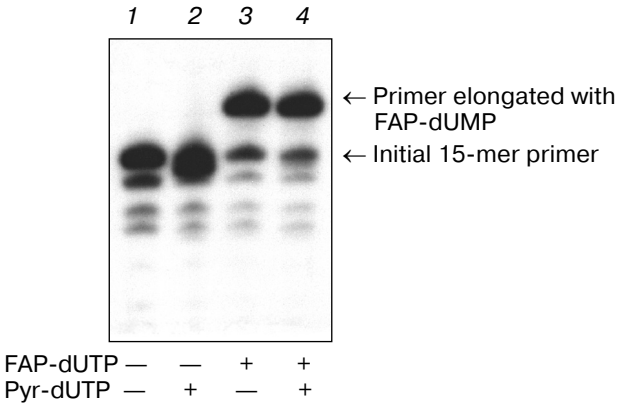

Fig. 2. Synthesis of photoreactive repair intermediates in nuclear extract. 5′-^{32}P-labeled DNA duplex was incubated in nuclear extract as described in "Materials and Methods". The reaction products were separated by electrophoresis in denaturing 20% polyacrylamide gel. Lanes: *1*) in the absence of dUTP analogs; *3*, *4*) in the presence of FAP-dUTP; *2*, *4*) in the presence of Pyr-dUTP.

interacting with photoreactive BER intermediates in nuclear extract [9, 10]. Earlier it was shown that the most efficient modification of DNA polymerases occurs when a photoreactive primer bearing 2,3,5,6-tetrafluoro-4-azidobenzoyl group at the 3′-end and Pyr-dUTP as sensitizer are used [29]. In this study, we for the first time used the FAP group as a photoreagent for sensitized modification; according to the recent data on photoaffinity labeling, the FAP group with the maximal efficiency cross-links with the protein targets under UV-irradiation in the wavelength region 300-320 nm [7]. To increase the yield of sensitized labeling, this reagent was used in the composition of a binary system. A typical labeling pattern of nuclear extract proteins using FAP-dUTP in the presence and in the absence of Pyr-dUTP under UV-irradiation at $\lambda = 365$ nm is shown in Fig. 4a, lanes *3-5*. As can be seen, under the photosensitized labeling conditions the amount of a covalent adduct with molecular mass 46 kD significantly increases (Fig. 4a, compare lanes *2* and *5*); this adduct may correspond to a product of labeling of DNA polymerase β with the DNA primer used. Being a specific ligand of DNA polymerases, under UV-irradiation at $\lambda = 365$ nm Pyr-dUTP efficiently induces covalent attachment of photoreactive primer adjacent to it in the active site of DNA polymerase. Under UV-irradiation at $\lambda = 365$ nm the intensity of the labeling products was low in the absence of Pyr-dUTP or in the presence of 1-pyrenebutyric acid, i.e., a potential sensitizer not bound with dNTP (Fig. 4a, lanes *3* and *4*); this indicates that photosensitized labeling depends on the presence of Pyr-dUTP. A radioactively labeled product with molecular mass ~97.4 kD is also detected in the absence of the photoreactive dNTP analog (Fig. 4, a and b, lane *1*). Consequently, this product is not obtained as the result of protein cross-linking with DNA containing a photoreactive dUMP residue. An analogous labeling pattern was obtained using another photoreagent, FAB-dUTP (Fig. 4b), but in this case the level of photosensitized labeling of DNA polymerase β was lower. We quantitatively evaluated efficiency of labeling of DNA polymerase β using a binary system of photoreagents. For FAP-dUTP, the level of photosensitized labeling of DNA polymerase β was ~60%, and for FAB-dUTP ~35%. It should be noted that using a binary system of photoreagents, the level of labeling of DNA polymerase β was higher under UV-irradiation at 365 nm than at 320 nm in the absence of Pyr-dUTP. However, the level of cross-linking with other nuclear extract proteins decreases in the case of labeling with the binary system (compare lanes *2* and *5* in Fig. 4, a and b); this indicates specificity of photosensitized labeling. When a FAP group-containing photoreagent is used, under UV-irradiation at $\lambda = 320$ nm more efficient modification of not only DNA polymerase β but also of other proteins is observed than in the case of FAB group-containing photoreagent (compare Figs. 4a and 4b, lanes *5*).

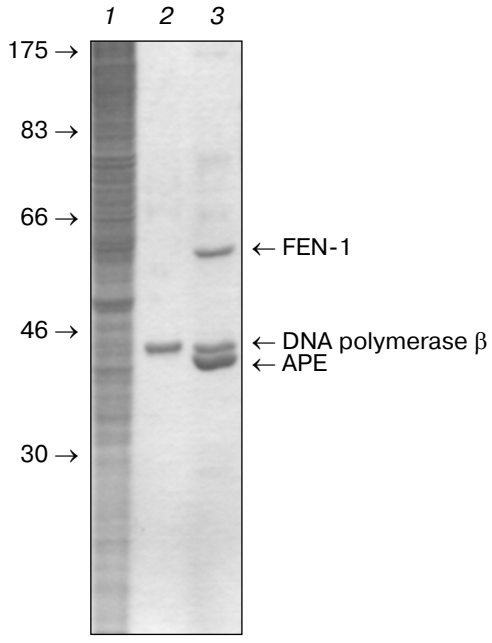

Fig. 3. Revealing of nuclear extract proteins by staining of the gel with Coomassie Brilliant Blue. Nuclear extract proteins (*1*) and purified recombinant: DNA polymerase β (*2*, *3*), FEN-1 (*3*), and APE (*3*) were separated by electrophoresis in 12% polyacrylamide gel in the presence of SDS with subsequent staining of the gel with Coomassie Brilliant Blue. Molecular masses of protein markers (kD) are given at the left.

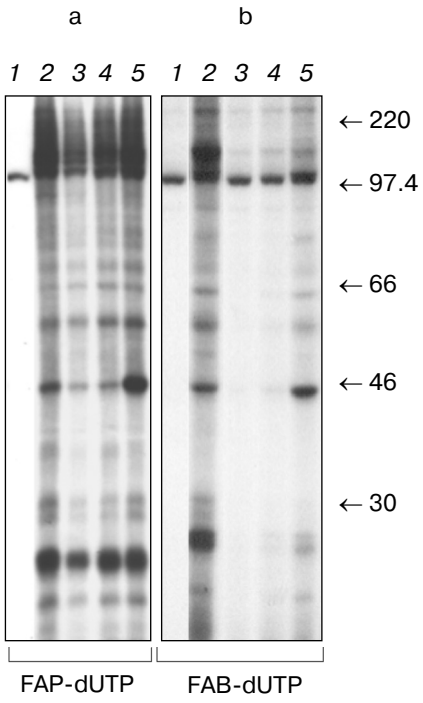

Fig. 4. Photoaffinity labeling of nuclear extract proteins. Photoreactive intermediate of DNA repair was synthesized with DNA polymerase of nuclear extract in the presence of FAP-dUTP (a) or FAB-dUTP (b) as described in "Materials and Methods". Control mixtures did not contain dUTP analog (*1*). The reaction mixtures were UV-irradiated at 320 nm ("direct" modification) (*1*, *2*) and at 365 nm in the presence of Pyr-dUTP ("sensitized" modification) (*5*), in the absence of Pyr-dUTP (*3*) or in the presence of 1-pyrenebutyric acid (*4*). The products of labeling were separated by electrophoresis in 12% polyacrylamide gel in the presence of SDS with subsequent autoradiography. Molecular masses (kD) of protein markers are given at the right.

Proteins of base excision repair such as polyadenylriboso-polymerase (PARP-1), flap-endonuclease (FEN-1), apurinic/apyrimidinic endonuclease (APE), and DNA polymerase β interacting with similar photoreactive intermediates in mouse fibroblast cell extract were recently identified by immunoprecipitation of DNA–protein adducts with specific antibodies [8]. We suggested that similar proteins can also interact with photoreactive BER intermediates in bovine testis nuclear extract. To prove this suggestion, after primer elongation by photoreactive FAB-dUTP analog but before UV-irradiation we added recombinant proteins of higher eukaryotes—PARP-1, FEN-1, APE, DNA polymerase β, and other proteins participating in DNA repair (RPA, DNA ligase I)—to nuclear extract. The data on separation of the labeling products thus obtained are presented in Fig. 5. As shown, addition to each protein results in change in the labeling pattern so that a covalent adduct corresponding in molecular mass to the product of combination of the added protein (DNA polymerase β, APE, FEN-1, and PARP-1) and 15-bp oligonucleotide becomes the main product of modification (Fig. 5, lanes *2-5*, respectively). Covalent attachment of the DNA residue of such dimensions to protein usually increases mobility of the labeling product by 6-7 kD. Mobility of these cross-linking products corresponds to that of similar proteins identified earlier in cell extract [8]. Addition of each excess protein results in decrease in the labeling intensity of other proteins of the extract; this indicates that proteins of the studied extract compete for interaction with photoreactive DNA repair intermediate. It should be noted that addition of low-molecular-weight proteins (DNA polymerase β, APE, and FEN-1) negligibly effected the level of PARP-1 modification (Fig. 5, lanes *2-4*); this is probably caused by high concentration of this protein in the extract [30], whereas addition of purified PARP-1 preparation to nuclear extract almost completely inhibits labeling of other proteins of nuclear extract (Fig. 5, lane *5*).

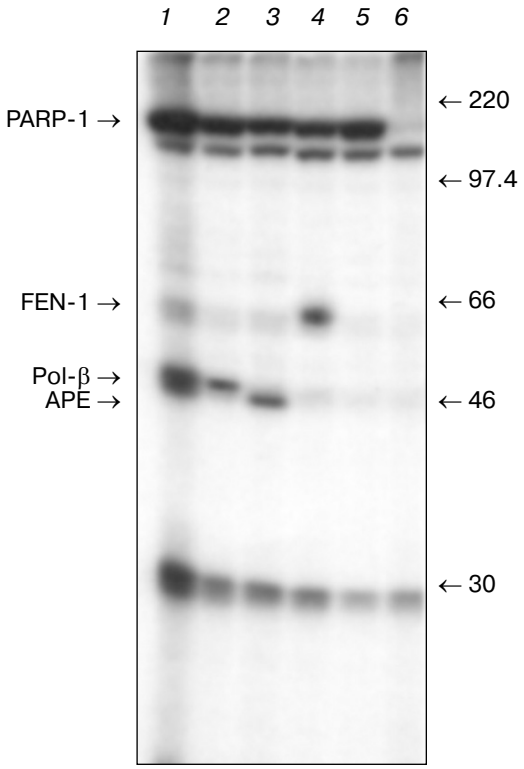

Fig. 5. Identification of nuclear extract proteins by addition of recombinant proteins of DNA repair. Photoreactive 5′-^{32}P-labeled DNA was obtained in the presence of FAB-dUTP using nuclear DNA polymerase. All reaction mixtures were UV-irradiated at 320 nm. Lanes: *1*) without addition of recombinant proteins; *2*) with addition of DNA polymerase β; *3*) with APE; *4*) with FEN-1; *5*, *6*) with addition of PARP-1 in the absence (*5*) or presence of NAD^+ (10^{-3} M) (*6*). The labeling products were separated by electrophoresis in 12% polyacrylamide gel in the presence of SDS with subsequent autoradiography. Molecular masses of protein markers (kD) are given at the right.

To confirm the nature of high-molecular-weight product, we performed experiments with addition of NAD^+ before UV-irradiation. This experiment was based on the fact that polyadenylation of PARP-1 occurs in the presence of NAD^+ and, consequently, mobility of the products of its cross-linking with DNA should change. As shown in Fig. 5, high-molecular-weight labeling product (~120 kD) almost disappears on addition of NAD^+; the amount of labeled products in the starting zone also increases (compare lanes *5* and *6*). Mobility of the products of DNA covalent binding to APE, DNA polymerase β, and FEN-1 does not change on addition of NAD^+ (data not presented here). Consequently, high-molecular-weight protein can be polyadenylated and corresponds to PARP-1, which is the main product of covalent binding to photoreactive BER intermediate in mouse fibroblast cell extract, as shown earlier [8]. Subsequent analysis demonstrated the role of PARP-1 as an activator of the long-patch base excision repair functioning in cooperation with FEN-1 [31].

Experiments on photosensitized labeling using a binary system of reagents reveal DNA polymerase β from the assembly of nuclear proteins as the main cross-linking product. Identification of the cross-linking product with molecular mass 46 kD to DNA polymerase β from bovine testis was proved by immunoprecipitation with specific antibodies (data not presented here). The data indicate that DNA polymerase β efficiently interacts with photoreactive BER intermediates in nuclear extract and seems to be the main enzyme participating in DNA resynthesis in long-patch base excision repair. From our viewpoint, for study of proteomic assemblies of DNA replication and repair, it is promising to use binary systems for detection of specific enzymes.

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