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Influence of Poly(ADP-ribose) Polymerase-1 and Its Apoptotic 24-kD Fragment on Repair of DNA Duplexes in Bovine Testis Nuclear Extract

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Abstract—Effects of exogenous proteins poly(ADP-ribose) polymerase-1 (PARP1) and its 24-kD proteolytic fragment (p24) on the repair of DNA duplexes containing a one nucleotide gap with furan phosphate or phosphate group at the 5′-end of the downstream primer were studied in bovine testis nuclear extract. These damaged DNAs are repaired by the long-patch or short-patch subpathways of base excision repair (BER), respectively. Exogenous PARP1 and p24 decreased the efficiency of gap filling DNA synthesis for both duplexes, but did not influence the ligation stage in the repair of DNA duplex by the short-patch subpathway. Under the same conditions, these proteins inhibited strand-displacement DNA synthesis and decreased the efficiency of the flap endonuclease 1 (FEN1)-catalyzed endonuclease reaction in the nuclear extract, blocking repair of DNA duplex by the long-patch subpathway. Addition of exogenous PARP1 and p24 also reduced the efficiency of UV light crosslinking of extract BER proteins to the photoreactive BER intermediates carrying a nick. Thus, PARP1 and p24 interact with DNA intermediates of BER and compete with nuclear extract proteins for binding to DNA. The interaction of PARP1 and p24 with DNA intermediates of the long-patch subpathway of BER resulted in inhibition of subsequent stages of the repair mediated by this mechanism. However, on recovery of the intact structure of DNA duplex by the short-patch subpathway, PARP1 and p24 suppressed the repair of the one nucleotide gap less efficiently and failed to influence the final stage of the repair, ligation.

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Key words: poly(ADP-ribose) polymerase-1, PARP1 apoptotic fragment, base excision repair, photomodification

Constant exposure of DNA to exogenous and endogenous genotoxic agents leads to instability of the cell genome [1]. Base excision repair (BER) is one of the main systems of repair responsible for replacement of damaged bases of DNA [2]. BER includes a succession of stages: removal of a damaged base, cleavage of the DNA chain with involvement of apurinic/apyrimidinic endonuclease (APE1), formation of the 3′-end of the DNA break to accept cellular DNA polymerases, synthesis of DNA, and ligation of nick [2].

Two main subpathways of BER differed: the short-patch subpathway (replacement of one nucleotide) and the long-patch subpathway (replacement of several nucleotides), which are mediated by BER enzymes and DNA replication factors [3, 4]. In addition to repair enzymes, BER involves other proteins, which can interact with DNA intermediates resulting in the repair and perform auxiliary or regulatory functions [5]. Poly(ADP-ribose) polymerase-1 (PARP1) is one of such proteins: it recognizes single-strand breaks in DNA caused by genotoxic agents (free radicals, ionizing radiation, monofunctional alkylating agents) and also in the course of BER [6]. The enzymatic activity of PARP1 is activated on interaction with DNA breaks: in the presence of NAD^+ a negatively charged polymer, poly(ADP-ribose), is synthesized, which is covalently bound to PARP1 or acceptor protein [7]. Autopoly(ADP-ribosyl)ation of PARP1 is suggested to initiate dissociation of the enzyme–DNA complex [8]. PARP1 and PARP1-dependent poly(ADP-

Abbreviations: APE1) apurinic/apyrimidinic endonuclease; BER) base excision repair; dRP) 2′-deoxyribose 5′-phosphate; FEN1) flap endonuclease 1; nt) nucleotides; PARP1) poly(ADP-ribose) polymerase-1; p24) 24-kD apoptotic fragment of PARP1; pF-N_1) 3-hydroxy-2-hydroxymethyl tetrahydrofuran 5′-phosphate mononucleotide; pol β) DNA polymerase β.

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ribosyl)ation in the case of local DNA damage can also promote activation of the repair enzyme complex or induce arrest of the cell cycle, providing time for effective repair [9, 10]. However, under conditions of massive DNA damage triggering programmed cell death, or apoptosis, PARP1 is cleaved to prevent its hyperactivation, which can cause a dramatic decrease in the NAD^+ concentration in the cell and depletion of ATP [10]. The catalytic activity of PARP1 is inactivated during apoptosis because of hydrolysis by caspases (with production of 89- and 24-kD fragments) [11]. The 89-kD fragment contains the catalytic and automodification domains, whereas the 24-kD fragment contains a DNA-binding motif ("zinc fingers") responsible for interaction with DNA breaks [12, 13]. It has been suggested that in the cell p24 can compete with intact PARP1 and the enzymes responsible for the DNA repair. Thus, the repair of oxy-radical damaged plasmid DNA in the extract from lymphoblastoid cells was inhibited in the presence of p24 [14]. Similar data were also obtained for γ-irradiated plasmid [15]. Thus, p24 effectively competes *in vitro* with the enzymes catalyzing BER, and it may be that generation of this PARP1 fragment during apoptosis really inhibits the repair of DNA.

This work continues a series of studies on proteins influencing BER. DNAs containing one nucleotide gaps and single-strand breaks are intermediates of BER, and both p24 and PARP1 are likely to be involved in BER via binding to DNA breaks. It has been shown earlier that PARP1 is a predominant product of covalent binding to nick-containing photoreactive DNA intermediates of the short-patch and long-patch subpathways of BER in mouse embryonic fibroblast cell extract and bovine testis nuclear extract [16, 17]. In the studied extracts, PARP1 was most intensively labeled when a photoreactive DNA substrate imitating an intermediate of the long-patch subpathway was used. The effect of PARP1 on the *in vitro* DNA synthesis catalyzed by DNA polymerase β (pol β) was studied using DNA substrates carrying a one nucleotide gap, and in the presence of PARP1 the inhibition of the strand-displacement DNA synthesis was significantly stronger than decrease in the efficiency of gap filling [18]. Poly(ADP-ribosyl)ation of PARP1 considerably decreased its inhibitory effect on DNA synthesis. Thus, the interaction of PARP1 with the single-strand break in the absence of NAD^+ inhibited the further DNA synthesis after the gap had been filled. Preliminary experiments revealed that the 24-kD fragment of PARP1 had a similar effect on the DNA synthesis catalyzed by pol β (unpublished data).

In the present work, we studied the influence of exogenous PARP1 and p24 on DNA synthesis and recovery of the intact structure of DNA duplexes repaired by the long-patch and short-patch subpathways of BER in bovine testis nuclear extract. DNA duplexes containing one nucleotide gaps with furan phosphate or phosphate group at the 5′-end of the downstream primer were used as DNA intermediates of BER. Addition of PARP1 or p24 decreased the efficiency of gap filling DNA synthesis but did not affect the final stage (ligation) during repair of DNA duplex by the short-patch subpathway of BER. In the presence of PARP1 or p24, both the strand-displacement synthesis and the endonuclease activity of flap endonuclease 1 (FEN1) of the nuclear extract were inhibited, and this prevented the DNA duplex repair by the long-patch subpathway. By using nick-carrying photoreactive DNA substrates, it was shown that the decrease in the efficiency and suppression of some stages in the repair of DNA duplexes is caused by competition of exogenous PARP1 and p24 with the extract proteins for binding to DNA.

MATERIALS AND METHODS

Materials. The following reagents were used: EDTA, NAD^+, N,N′-methylenebisacrylamide, Tris, IPTG (isopropyl-β-D-thiogalactoside), and glycerol from Sigma (USA); β-mercaptoethanol, bromophenol blue, xylene cyanole, and formamide from Fluka (Switzerland); acrylamide from Helicon (Russia); dATP, dCTP, dGTP, and dTTP from Biosan (Russia); [γ-^{32}P]ATP (185 TBq/mmol), [α-^{32}P]ATP (110 TBq/mmol), and [α-^{32}P]dATP (110 TBq/mmol) produced by the Biotechnology Laboratory, Institute of Chemical Biology and Fundamental Medicine, Siberian Division of the Russian Academy of Sciences (Russia); TEMED (N,N,N′,N′-tetramethylethylenediamine) and urea from Merck (Germany); High Range markers of protein molecular mass from Amersham (USA). Other reagents produced in Russia were of special purity qualification. The synthesis and photochemical properties of the dCTP analog exo-N-[4-(4-azido-2,3,5,6-tetrafluorobenzylidene hydrazinocarbonyl)-butylcarbamoyl]-2′-deoxycytidine-5′-triphosphate (FABGdCTP) were described earlier [19].

DNA substrates. All synthetic oligodeoxynucleotides were obtained from Oligos Etc, Inc. (USA). The oligodeoxynucleotides were labeled with [^{32}P]phosphate at the 5′-end with involvement of T4 polynucleotide kinase and purified by electrophoresis in denaturing polyacrylamide gel containing 7 M urea [20]. DNA duplexes were prepared by equimolar mixing complementary oligodeoxynucleotides in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The resulting reaction mixtures were heated to 90°C for 3 min and then slowly cooled to room temperature. The resulting DNA duplexes were analyzed by electrophoresis in 10% polyacrylamide gel under native conditions.

Enzymes and extracts. Benzonase was from Merck (Germany). Rat recombinant DNA polymerase β (EC 2.7.7.7) was purified as described in [21]. Plasmids pET 32 containing cDNA of the human PARP1 gene or

cDNA of its 24-kD fragment were kindly presented by Dr. M. C. Satoh (Laval University, Canada). Human recombinant poly(ADP-ribose) polymerase-1 (EC 2.4.2.30) was purified according to [18]. The p24 fragment was expressed in the system of *E. coli* (strain BL21 DE3 (pLys E)) and purified similarly to isolation of PARP1 [18]. The bovine testis nuclear extract was prepared as described in [22]. Protein concentrations in the extract were determined by the Bradford method [23].

DNA repair assay. The DNA repair reactions were carried out in the standard reaction mixture (10 μl) containing the nuclear extract proteins (1.8 mg/ml), 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 7 mM β-mercaptoethanol, 10 mM $MgCl_2$, dATP, dGTP, dTTP, and dCTP (10 μM each), 0.5 mM NAD^+, 5 mM ATP, and 50 nM DNA substrate. The FEN1 cleavage assay in the reconstituted system was performed in the standard reaction mixture (10 μl) containing FEN1 (0.5 μM), pol β (0.05 μM), 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 7 mM β-mercaptoethanol, 10 mM $MgCl_2$, dATP, dGTP, dTTP, and dCTP (10 μM each), and 50 nM DNA*-1. The concentrations of PARP1 and p24 were 0.5 and 3.0 μM, respectively. The reaction was initiated by addition of the four dNTPs. The endonuclease reaction in the presence of ATP and NAD^+ was initiated by simultaneous addition of NAD^+ to 0.5 mM, ATP to 5.0 mM, and the four dNTPs. The reaction mixtures were incubated at 37°C for 5-20 min, and the reaction was stopped by addition of 20 μl of mixture containing 90% formamide, 50 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanole. The reaction mixtures were heated for 5 min, and the reaction products were analyzed by electrophoresis in 20% polyacrylamide gel containing 7 M urea, 89 mM Tris, 89 mM boric acid, and 2 mM EDTA (pH 8.0), with subsequent radioautography or scanning with Molecular Imager (BioRad, USA). The electrophoresis was performed on 20 × 20 × 0.04 cm plates at the voltage of 50 V/cm.

Synthesis of photoreactive BER intermediates and photoaffinity modification. Photoreactive DNA substrates were synthesized in 100 μl of reaction mixture containing DNA-F or DNA-P (0.1 μM), 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 50 nM pol β, 70 μM FABGdCTP, and [α-^{32}P]dATP (10 MBq). The mixtures were incubated for 45 min, then the reaction was stopped by addition of 10 volumes of 2% $LiClO_4$ solution in acetone, and the precipitate was centrifuged and dissolved in buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. The photochemical crosslinking reaction mixture (20 μl) contained the nuclear extract proteins (1.8 mg/ml), 50 nM DNA substrate, 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 10 mM $MgCl_2$, in the presence of 5 mM ATP and 0.5 mM NAD^+ where indicated. The reaction mixture was incubated for 3-10 min at 37°C and UV-radiated with a DRK-120 high-pressure mercury lamp in a VIO-1 illuminator (LOMO, Russia), at the distance of 110 mm, in the range of 313-365 nm, with a UFS-6 photofilter, at incident light intensity $I = 8 \cdot 10^{14}$ quanta·sec^{-1}·cm^{-2}. The modification products were separated by electrophoresis in 10% SDS-polyacryl amide gel by the Laemmli method [24]. The distribution of radioactivity in the gel was visualized as described above.

Preparation of intermediate DNA ligase-[^{32}P]AMP in nuclear extract. The reaction mixture (20 μl) containing the nuclear extract proteins (1.8 mg/ml), 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 10 mM $MgCl_2$ was incubated in the presence of 1 μM [α-^{32}P]ATP for 10 min [23]. When analyzing the intermediate DNA ligase-[^{32}P]AMP during the repair of DNA duplexes, after incubation with [α-^{32}P]ATP, the reaction mixture was supplemented with 50 nM DNA-1 (or DNA-2), 10 μM four dNTPs, and 5 mM ATP and incubated further for 15 min [25]. Before application onto the gel, the reaction mixtures were heated for 15 min at 65°C. The subsequent analysis was performed as described above.

RESULTS

Influence of exogenous PARP1 and p24 on repair in nuclear extract of DNA duplexes containing a one nucleotide gap. To study repair in nuclear extract, DNA duplexes containing a one nucleotide gap in the middle of one chain were used. Oligonucleotide flanking the gap from the 5′-end contained at its 5′-end either the tetrahydrofuran phosphate group (DNA-1) or phosphate group (DNA-2) (table). DNA-2 can be considered as an intermediate of the short-patch subpathway of BER and DNA-1 as an intermediate of the long-patch subpathway of BER, because the tetrahydrofuran phosphate group (an analog of the dRP group) cannot be removed by the lyase activity of pol β [26]. In the presence of dNTPs, the repair of these substrates catalyzed by the extract proteins mainly resulted in the product of DNA synthesis corresponding to one nucleotide gap filling. This product was 60% for DNA-1 and 70% for DNA-2 (Fig. 1a, lanes *1-4*, *13-16*). The further strand-displacement synthesis was less effective (Fig. 1a, lanes *5* and *16*). The addition of PARP1 or p24 decreased the gap filling efficiency, and at the protein/DNA ratio of 10 : 1 for PARP1 and 60 : 1 for p24 the strand-displacement synthesis was virtually fully suppressed (Fig. 1a, lanes *5-12* and *17-24*). It is supposed that in the presence of NAD^+, poly(ADP-ribosyl)ation of PARP1 results in dissociation of the PARP1 complex with DNA [7, 8]. The addition of NAD^+ to the PARP1-containing mixtures increased the rate of the one nucleotide gap filling DNA synthesis, but the total efficiency of DNA synthesis did not reach the level observed in the absence of exogenous PARP1. It was revealed by the decreased length of the average statistical product of the reaction and the lesser extent of elongation of the initial primer (Fig. 1b, lanes *9-12* and *21-24*; compare with Fig.

1a, lanes *9-12* and *21-24*). NAD^+ itself had virtually no influence the efficiency of DNA synthesis in the extract (compare Fig. 1b, lanes *1-4* and Fig. 1a, lanes *1-4*) and displayed no additional effect in the reaction mixture containing p24 (compare Fig. 1a, lanes *5-8*, *17-20* and Fig. 1b, lanes *5-8*, *17-20*).

Depending on the BER subpathway, DNA synthesis can be catalyzed by either pol β or pol δ(ε) [2]. According to the literature, pol β, in contrast to pol δ(ε), effectively incorporates ddNTP [27]. We have studied DNA synthesis catalyzed by the extract at varied ratios of ddCTP/dCTP (3 : 7, 6 : 4, and 9 : 1) and the total concentration of the dCTP and ddCTP was equal to 10 μM (Fig. 1c, lanes *3-5* and *8-10*). According to the nucleotide sequence of the template used, the incorporation of ddCMP had to lead to accumulation of a product corresponding to the one nucleotide gap filling (16 nt). In the presence of 90% of ddCTP, the product 16 nt in length dominated and the further elongation of the primer was inhibited (Fig. 1c, compare lanes *2* and *5*, *7* and *10*). It is possible that on the repair of both DNA duplexes in the extract, pol β is responsible for the gap filling DNA synthesis and the decrease in the efficiency of DNA synthesis at this stage in the presence of p24 or PARP1 was caused by competition of the exogenous proteins with this polymerase for the binding of DNA substrate.

DNA repair is completed with involvement of DNA ligases in the presence of ATP [2]. On addition of ATP, a 34-nt product was observed (Fig. 2a, lanes *1-4* and *13-16*). But the efficiency of repair of DNA-1 was consider-

Nucleotide sequences, structures, and designations of DNA substrates

Nucleotide sequence	Designation
5′-F\ 5′-[^{32}P]-CTGCAGCTGATGCGC GTACGGATCCCCGGGTAC-3′ 3′-GACGTCGACTACGCG**G**CATGCCTAGGGGCCCATG-5′	DNA-1
5′-P\ 5′-[^{32}P]-CTGCAGCTGATGCG C GTACGGATCCCCGGGTAC-3′ 3′-GACGTCGACTACGCG**G**CATGCCTAGGGGCCCATG-5′	DNA-2
5′-[^{32}P] F\ 5′-CTGCAGCTGATGCGC GTACGGATCCCCGGGTAC-3′ 3′-GACGTCGACTACGCG**G**CATGCCTAGGGGCCCATG-5′	DNA*-1
5′-F\ 5′-CTGCAGCTGATGCGC **C*A^{32}P** GTACGGATCCCCGGGTAC-3′ 3′-GACGTCGACTACGCG **G –T –** CATGCCTAGGGGCCCATG-5′	DNA$^{(FABG)}$F
5′-P\ 5′-CTGCAGCTGATGCGC **C*A^{32}P** GTACGGATCCCCGGGTAC-3′ 3′-GACGTCGACTACGCG **G–T–** CATGCCTAGGGGCCCATG-5′	DNA$^{(FABG)}$P
5′-F\ 5′-CTGCAGCTGATGCGC GTACGGATCCCCGGGTAC-3′ 3′-GACGTCGACTACGCG **GT**CATGCCTAGGGGCCCATG-5′	DNA-F
5′-P\ 5′-CTGCAGCTGATGCGC GTACGGATCCCCGGGTAC-3′ 3′-GACGTCGACTACGCG **GT**CATGCCTAGGGGCCCATG-5′	DNA-P

Note: F, 3-hydroxy-2-hydroxymethyl tetrahydrofuran with 5′-phosphate; P, phosphate; C*, FABGdCTP (exo-N-[4-(4-azido-2,3,5,6-tetrafluorobenzylidene hydrazinocarbonyl)-butylcarbamoyl]-2′-deoxycytidine-5′-triphosphate).

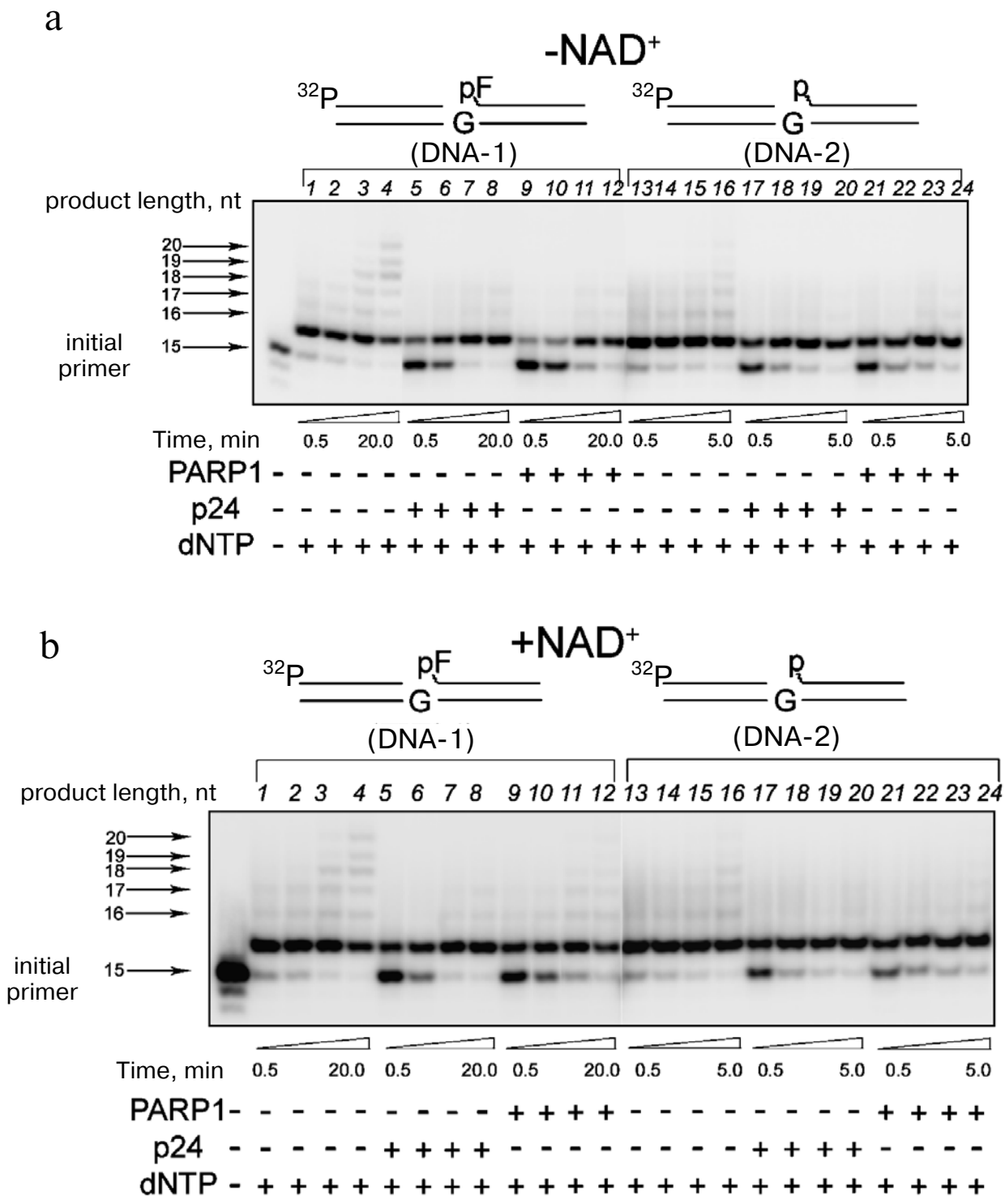

Fig. 1. Influence of exogenous PARP1 and p24 on DNA synthesis catalyzed by bovine testis nuclear extract (a), the same in the presence of NAD^+ (b), and also DNA synthesis catalyzed by nuclear extract in the presence of ddCTP (c). a) The reaction was carried out in the standard mixture containing DNA-1 (lanes *1-12*) or DNA-2 (lanes *13-24*) in the presence of PARP1 (lanes *9-12* and *21-24*) or p24 (lanes *5-8* and *17-20*). b) The reaction was carried out in the standard mixture containing DNA-1 (lanes *1-12*) or DNA-2 (lanes *13-24*) and NAD^+ (0.5 mM) in the presence of PARP1 (lanes *9-12* and *21-24*) or p24 (lanes *5-8* and *17-20*). c) The reaction was carried out in the standard mixture containing DNA-1 (lanes *1-5*) or DNA-2 (lanes *6-10*) in the presence of four dNTPs (10 μM each) (lanes *2*, *7*) or the mixture of dATP, dGTP, dTTP (10 μM each), and varied ratio of concentrations of dCTP and ddCTP: dCTP (7 μM) and ddCTP (3 μM) (lanes *3*, *8*); dCTP (4 μM) and ddCTP (6 μM) (lanes *4*, *9*); dCTP (1 μM) and ddCTP (9 μM) (lanes *5* and *10*).

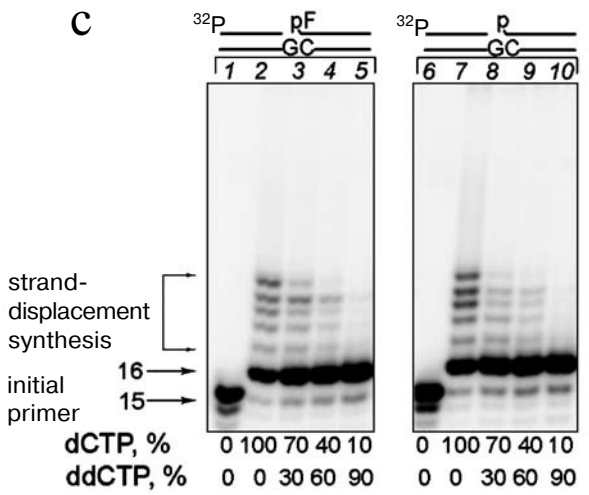

Fig. 1. (Contd.)

ably lower, only 35% of the DNA-1 was repaired with forming full-length product (34 nt) within 20 min, while for DNA-2 the amount of product (34 nt) runs up to 90% within 5 min (Fig. 2a, lanes *4* and *16*). Addition of PARP1 or p24 had virtually no influence of the ligation efficiency on the repair of DNA-2 (Fig. 2a, lanes *17-20* and *21-24*). As to the case of DNA-1, p24 and PARP1 inhibited the formation of the ligation product (34 nt) (Fig. 2a, lanes *5-8*, *9-12*). Addition of NAD^+ into the system containing exogenous PARP1 partially recovered the efficiency of DNA synthesis (compare lanes *9-12* in Figs. 2a and 2b), but, in general, the formation of the intact structure of DNA duplex was suppressed during the DNA-1 repair (Fig. 2b, lanes *1-4* and *9-12*).

DNA-1 has to be repaired with involvement of FEN1, which cleaves the flapping 5′-end of DNA with the furan phosphate residue being formed during the strand-displacement DNA synthesis [4, 28]. Moreover, FEN1 can excise the first nucleotide with the 5′-furan phosphate group at the 5′-end of the primer flanking the nick [29]. On using DNA*-1 substrate similar to DNA-1 (table) where downstream primer were radiolabeled at the 5′-end, the main excision product corresponded to single nucleotide with the furan phosphate group at the 5′-end ($pF\text{-}N_1$) (Fig. 3a, lanes *1-3*). Under conditions of DNA synthesis, the content of $pF\text{-}N_1$ was 27% of the total amount of endonuclease cleavage products and increased to 40% on the addition of ATP and NAD^+. The total amount of products with length of 3-7 nucleotides (from $pF\text{-}N_3$ to $pF\text{-}N_7$) did not exceed 22%.

Our experiments demonstrated that DNA*-1 was repaired via the mechanism of "gap translation" which has been earlier shown *in vitro* in a reconstituted BER system [30]: on formation of a nick-containing DNA intermediate, FEN1 stimulated the low efficiency of DNA synthesis on this substrate of pol β by translation of the one nucleotide gap, removing a nucleotide from the 5′-end of the break and creating a new mononucleotide gap which was filled by pol β [30].

The data in Fig. 3b (lanes *1* and *2*) demonstrate FEN1 exonucleolytic products for DNA*-1 in the presence or absence of pol β in the reconstituted system. As in the extract, the endonuclease cleavage mainly resulted in $pF\text{-}N_1$, and its amount was 45% in the absence of pol β and 73% under conditions of DNA synthesis. In the presence of pol β, the amount of resulting products with length from $pF\text{-}N_2$ to $pF\text{-}N_9$ did not exceed 18.5% of the initial primer.

For DNA*-1, exogenous PARP1 and p24 decreased the efficiency of the endonuclease reaction in the extract mainly by suppression of $pF\text{-}N_1$ formation (Fig. 3a, lanes *4*, *5* and *7*, *8*). The addition of NAD^+ into the system containing exogenous PARP1 did not completely recover the efficiency of the endonuclease cleavage in the course of DNA*-1 repair (Fig. 3a, compare lanes *3* and *6*). Both p24 and PARP1 similarly influenced this process in the reconstituted system with respect to FEN1 (Fig. 3b, compare lanes *1* and *3*, *7*) and also when FEN1 and pol β were added together (Fig. 3b, compare lanes *2* and *4*, *8*). Note, that in both the reconstituted BER system and the extract the addition of NAD^+ to the PARP1-containing system also did not fully recover the efficiency of the endonuclease reaction (Fig. 3b, compare lanes *1*, *2* and *5*, *6*). Consequently, in both the reconstituted system and the extract poly(ADP-ribosyl)ation of exogenous PARP1 failed to completely recover efficiency of the repair of DNA duplexes.

Interaction of nuclear extract proteins with photoreactive DNA intermediates of BER and influence of ATP and NAD^+ on this process. To compare the interaction of the extract proteins with DNA intermediates produced during the repair of DNA duplexes by the short-patch and long-patch subpathways of BER, we used DNAs with a photoreactive group in the region of the 3′-end of the nick. The BER enzymes could be selectively labeled in both mouse embryonic fibroblasts cell extract (MEF) [16, 31] and the bovine testis nuclear extract [17] with nicked DNA intermediates containing a photoreactive nucleotide at the 3′-end of margin of a nick.

In the present work we used DNA substrates being presynthesized *in vitro* by pol β and containing the photoreactive FABGdCMP residue in the second position from the 3′-end of the upstream primer; to further characterize the cross-linking products, the radioactive residue dAMP was introduced directly onto the 3′-end. The downstream primer of the nick carried furan phosphate ($DNA^{(FABG)}F$) or phosphate ($DNA^{(FABG)}P$) group (table) at the 5′-margin. Thus, $DNA^{(FABG)}F$ imitated DNA intermediate of the long-patch subpathway of BER after the gap filling, and $DNA^{(FABG)}P$ imitated the penultimate product of the short-patch subpathway before the nick ligation.

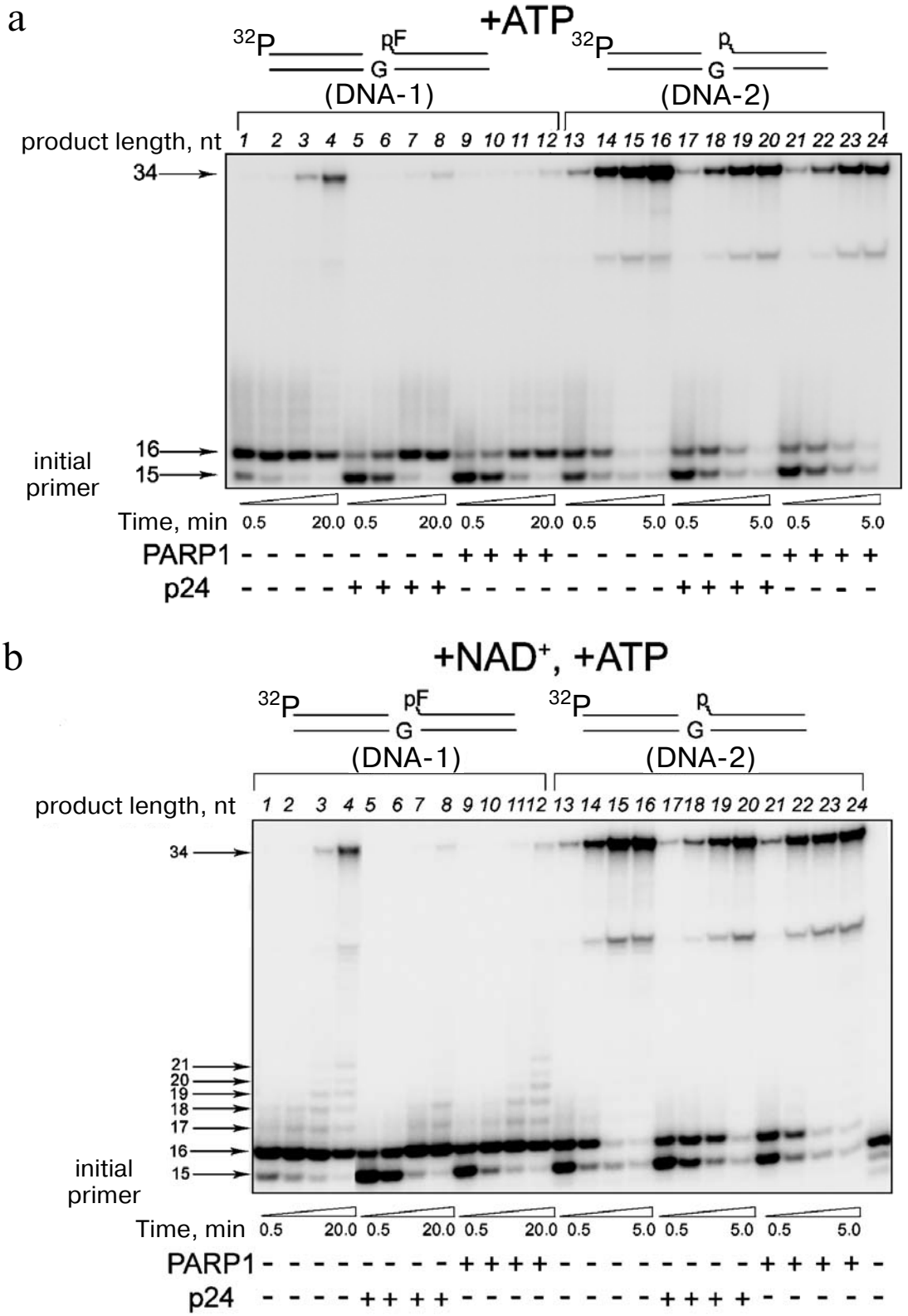

Fig. 2. Influence of exogenous PARP1 and p24 on DNA repair catalyzed by nuclear extract in the presence of ATP (a) and ATP and NAD^+ (b). a) The reaction was carried out in the standard mixture containing DNA-1 (lanes *1-12*) or DNA-2 (lanes *13-24*), ATP (5 mM) and in the presence of PARP1 (lanes *9-12* and *21-24*) or p24 (lanes *5-8* and *17-20*). b) The reaction was carried out in the standard mixture containing DNA-1 (lanes *1-12*) or DNA-2 (lanes *13-24*), ATP (5 mM), NAD^+ (0.5 mM) and in the presence of PARP1 (lanes *9-12* and *21-24*) or p24 (lanes *5-8* and *17-20*).

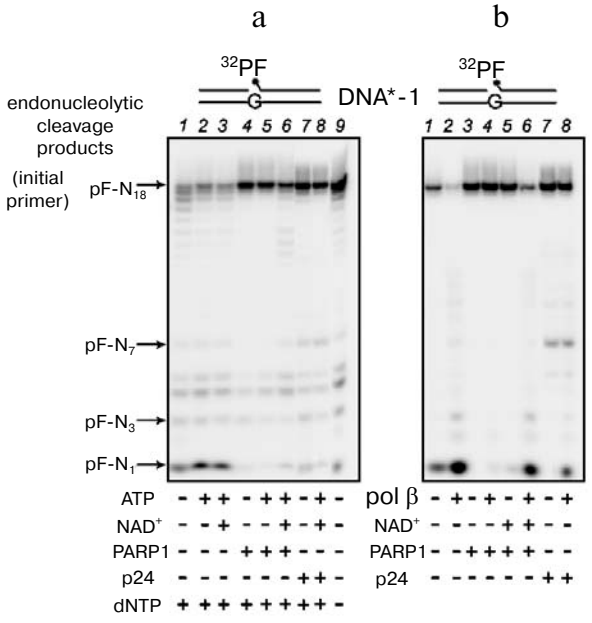

Fig. 3. Influence of exogenous PARP1 and p24 on formation of exonucleolytic cleavage products in the course of DNA*-1 repair catalyzed by nuclear extract (a) and the reconstituted BER system (b). a) The reaction was carried out for 20 min at 37°C in the standard reaction mixture containing 50 nM DNA*-1 in the presence of PARP1 (lanes *4-6*) or p24 (lanes *7* and *8*) and ATP (5 mM) and NAD$^+$ (0.5 mM) where indicated. b) In the reconstituted BER system the reaction was carried out in the standard reaction mixture containing DNA*-1, FEN1 (0.5 μM), pol β (0.05 μM), and NAD$^+$ (0.5 mM) where indicated, in the presence of PARP1 (lanes *3-6*) or p24 (lanes *7* and *8*).

The photoinduced binding of DNA$^{(FABG)}$F and DNA$^{(FABG)}$P to the extract proteins resulted in labeling of a limited number of proteins, and the cross-linking products showed the same electrophoretic mobilities for both DNA substrates (Fig. 4a, lanes *5* and *6*). On addition of recombinant proteins of BER into the reaction mixture, the electrophoretic mobilities of products of exogenous PARP1 and pol β labeling corresponded to 120- and 46-kD products of modification of the extract proteins (Fig. 4a, compare lanes *1-4* and *5*, *6*). The 120- and 46-kD products of labeling were earlier identified as PARP1 and pol β, respectively [31]. Note that covalent binding of DNA to various amino acid residues of the same protein can give products with different electrophoretic mobilities [32]. Therefore, to more correctly compare the cross-linked products of extract proteins and ones of the recombinant BER proteins to DNA$^{(FABG)}$F and DNA$^{(FABG)}$P, we used nuclease hydrolysis to remove DNA in the DNA–protein adducts. In this case, the radiolabeled nucleotide near the site of protein–nucleic acid crosslinking is protected by the protein against the nuclease hydrolysis. The removal of DNA increased the electrophoretic mobilities of the labeled products of proteins and intensities of the cross-linked products migrating at 110-, 40-, and 30-kD. Moreover, intensities of the bands corresponding to minor products were decreased (Fig. 4a, lanes *7*, *8*, *9-12*). After the treatment with nuclease, only products of labeling of recombinant PARP1 and pol β coincided in the electrophoretic mobilities with the products of modification of proteins with apparent molecular mass of 110 and 40 kD visualized in the extract (Fig. 4a, lanes *7*, *8* and *9-12*).

In the presence of low-molecular-mass substrates of ATP and NAD$^+$, the labeling pattern of the extract proteins interacting with the structures of DNA$^{(FABG)}$F and DNA$^{(FABG)}$P changed. Thus, for the two DNA duplexes, the addition of ATP decreased the yield of the 40-kD labeling product corresponding to pol β and increased intensities of the bands of labeling of PARP1 and the 100- and 90-kD extract proteins (protein-1 and protein-2, respectively) (Fig. 4b, compare lanes *4* and *5*, *13* and *14*). By the molecular mass, the labeling product "protein-1" may correspond to DNA ligase III, which completes BER by sealing a nick in the presence of ATP [23]. To identify DNA–protein adducts of DNA ligases, we used the ability of these enzymes to produce a DNA ligase-[^{32}P]AMP intermediates that is stable in the absence of DNA substrate [25]. Separation by SDS-PAGE of the extract proteins being incubated with [α-^{32}P]ATP allowed us to visualize in extract adenylated proteins with apparent molecular masses of 120, 100, and 70 kD (Fig. 4b, lane *19*) which may be [^{32}P]AMP intermediates of eukaryotic DNA ligases: DNA ligase I, DNA ligase III, and DNA ligase II, respectively. Simultaneous addition of dNTP, ATP, and DNA-1 (or DNA-2) decreased the intensity of the band corresponding to the 100-kD [^{32}P]AMP–protein intermediate (Fig. 4b, lanes *20* and *21*) that seemed to be due to use of the adenylated enzyme for nick sealing during repair. It is possible that in this extract DNA ligase III mainly catalyzed the ligation of the nick, which is formed at the terminal stage during repair both with DNA-1 and DNA-2.

It should be noted that the modification of the extract proteins with DNA$^{(FABG)}$F or DNA$^{(FABG)}$P in the presence of ATP led to an increase in the labeling level of protein-1, which had the same electrophoretic mobility as [^{32}P]AMP–DNA ligase III of the nuclear extract, and this was likely to be due to the more efficient interaction of this enzyme with DNA in the presence of ATP. The simultaneous addition of NAD$^+$ and ATP resulted in disappearance of the product of modification of endogenous PARP1, whereas the set of other products of labeling was unchanged (Fig. 4b, compare lanes *4* and *6*, *13* and *15*). Thus, the sets of products of labeling were not considerably different during the interaction of the extract proteins with the photoreactive BER intermediates DNA$^{(FABG)}$F and DNA$^{(FABG)}$P. Functional analysis also indicated the identity of the sets of enzymes catalyzing the repair of DNA duplexes (DNA-1 and DNA-2) by the long-patch and short-patch subpathways of BER.

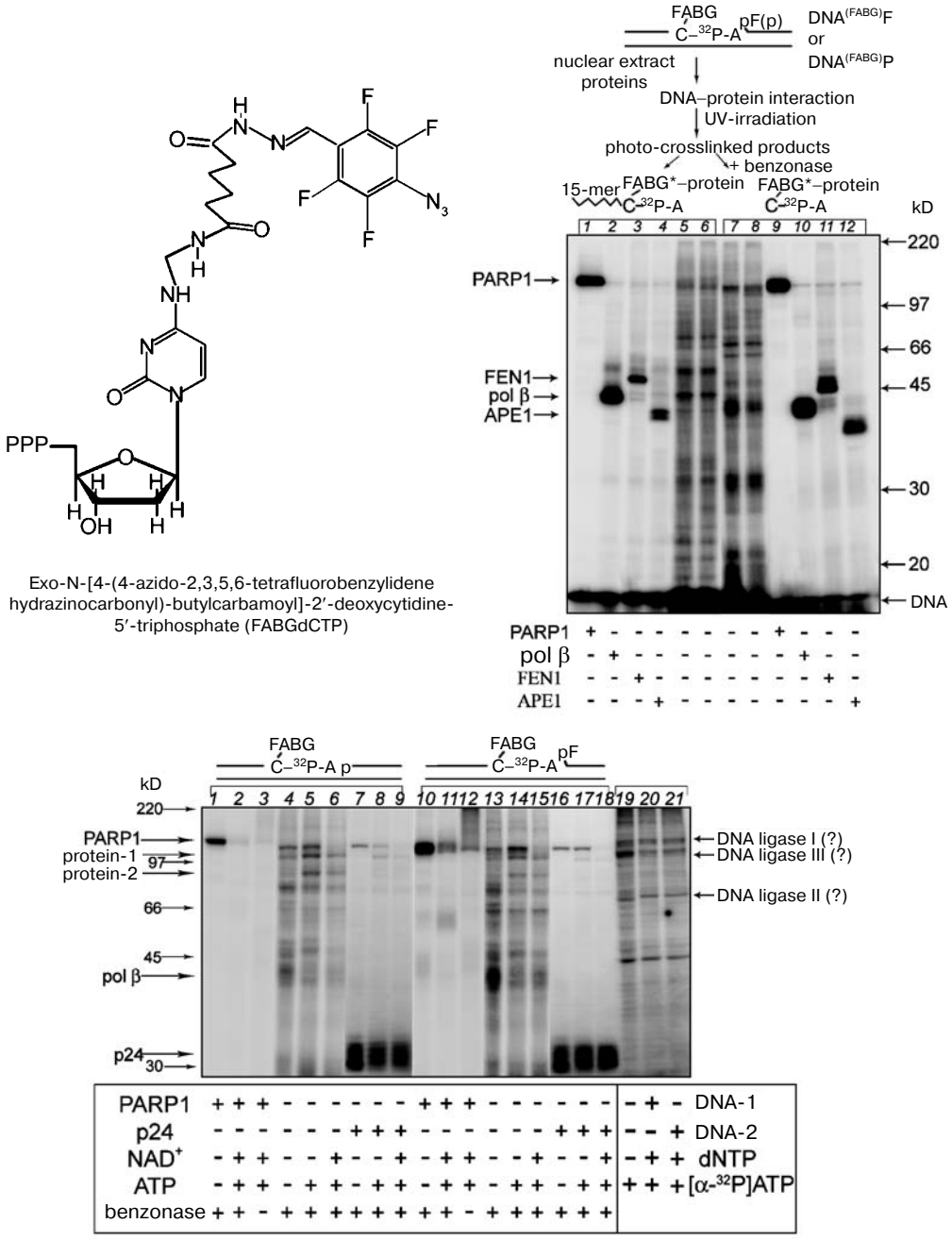

Fig. 4. Structure of the photoreactive dCTP analog (FABGdCTP) (a) and comparative analysis of cross-linked products of the nuclear extract proteins and the extra added recombinant proteins to photoreactive BER intermediates (b). a) The products of labeling of the extract proteins with DNA$^{(FABG)}$P (lane *6*) and DNA$^{(FABG)}$F (lanes *1-5*) with adding of pol β (5 μM) (lane *2*), APE1 (5 μM) (lane *4*), PARP1 (0.5 μM) (lane *1*), and FEN1 (5 μM) (lane *3*) were separated by 10% SDS-PAGE. Comparative analysis of photocross-linked products of the extract proteins and the recombinant proteins after nuclease treatment of DNA–protein adducts was carried out. The products of labeling of the extract proteins with DNA$^{(FABG)}$P (lane *8*) and DNA$^{(FABG)}$F (lanes *9-12*, *7*) with adding of recombinant proteins with pol β (lane *10*), APE1 (lane *12*), PARP1 (lane *9*), and FEN1 (lane *11*) were treated with benzonase and separated by 10% SDS-PAGE. Molecular masses of the markers are indicated to the right. b) Comparative analysis of products of labeling of the nuclear extract proteins in the presence of exogenous PARP1 or p24. DNA$^{(FABG)}$F (lanes *10-18*) or DNA$^{(FABG)}$P (lanes *1-9*) were incubated with the extract proteins (lanes *4-6* and *13-16*) in the presence of PARP1 (lanes *1-3* and *10-12*) or p24 (lanes *7-9* and *16-18*), with addition of ATP and NAD$^+$ where indicated. Cross-linked products of nuclear proteins were treated with benzonase and separated by 10% SDS-PAGE. DNA ligases-[α-^{32}P]AMP of the nuclear extract were analyzed in the reaction mixture, as described in "Materials and Methods", in the presence of 1 μM [α-^{32}P]ATP (lanes *19-21*) and 50 nM DNA substrate and 10 μM dNTP (DNA-1, lane *20*; DNA-2, lane *21*). The extract proteins were incubated with [α-^{32}P]ATP for 10 min to form DNA ligases-[α-^{32}P]AMP, and separated by 10% SDS-PAGE. Molecular masses of the markers are indicated to the left.

Interaction of nuclear extract proteins with photoreactive BER substrates in the presence of exogenous PARP1 and the 24-kD apoptotic fragment. A decrease in the efficiency of separate stages of DNA-1 and DNA-2 repair in the presence of p24 or PARP1 may be considered as a result of competition between these proteins and enzymes of the extract for the binding of DNA substrate. This hypothesis is in agreement with the initial model of the PARP1 functioning on the repair of DNA, which has been proposed based on the NAD^+-dependence of BER [7, 8]. The 24-kD fragment possessing the DNA-binding activity but lacking the catalytic function can also compete with the enzymes responsible for the repair [14, 15]. Therefore, we have performed photoaffinity modification of the extract proteins by $DNA^{(FABG)}F$ and $DNA^{(FABG)}P$ (table) in the presence of exogenous p24 or PARP1. The addition of PARP1 or p24 into the reaction mixture significantly suppressed the labeling of the extract proteins (Fig. 4b, compare lanes *4* and *1*, *7*; *13* and *10*, *16*), and the major product of the modification corresponded to the exogenous protein (PARP1 or p24). However, p24 did not influence the modification level of endogenous PARP1 (Fig. 4b, lanes *4* and *7*, *13* and *16*). The decrease in the modification level of the extract proteins on addition of PARP1 (or p24) at the protein/DNA ratio of 10 : 1 (or 60 : 1) is consistent with data of kinetic analysis which, at the same ratio of exogenous proteins and DNA substrate, have shown the inhibition of the strand-displacement synthesis for DNA-1 and DNA-2 and the suppressed recovery of the intact structure of DNA-1 when ATP, or ATP and NAD^+ were added simultaneously with dNTPs.

On the modification of the extract proteins by $DNA^{(FABG)}F$ and $DNA^{(FABG)}P$ in the presence of p24 and ATP, we observed predominant cross-linking of p24, and only products of the PARP1 and protein-1 modification, presumably DNA ligase III, could be clearly detected among the extract proteins (Fig. 4b, compare lanes *5* and *8*, *14* and *17*). In the case of $DNA^{(FABG)}F$, the addition of ATP did not change the labeling level of the proteins p24 and endogenous PARP1, but for $DNA^{(FABG)}P$ their modification levels were decreased (Fig. 4b, compare lanes *7* and *8*). After separation of the UV-irradiated reaction mixture by denaturing polyacrylamide gel, ligated product (34-nt) was observed.

Thus, the presence of p24 did not prevent the sealing of nicked $DNA^{(FABG)}P$ by DNA ligase. Generation of the ligation product where the photoreactive residue was in the intact chain of DNA decreased the modification level of endogenous PARP1 and p24 specifically interacting with nick-containing DNA. The product corresponding to labeling of endogenous PARP1 was not observed on simultaneous addition of p24, ATP, and NAD^+ in the case of both photoreactive DNA substrates. For $DNA^{(FABG)}P$, as in the presence of ATP, the labeling level of p24 and the extract protein-1 were decreased (Fig. 4b, lanes *9* and *18*). In the presence of NAD^+, poly(ADP-ribosyl)ation of endogenous PARP1 seemed to decrease its affinity for $DNA^{(FABG)}F$, and no products of its modification were observed. However, under these conditions, p24 interacts with DNA substrate, and this binding could suppress the repair.

The addition of ATP or ATP in combination with NAD^+ had a similar effect on the labeling of the extract proteins by $DNA^{(FABG)}F$ and $DNA^{(FABG)}P$ duplexes in the presence of exogenous PARP1. Thus, in the case of $DNA^{(FABG)}P$, the level of PARP1 labeling was decreased (Fig. 4b, lanes *2* and *3*) due to forming of an intact DNA chain carrying inside a photoreactive residue. However, on the modification of the extract proteins by $DNA^{(FABG)}F$ in the presence of exogenous PARP1, ATP, and NAD^+, high-molecular-mass products of labeling with the apparent molecular weights from 100 to 220 kD were observed, which could correspond to modification of poly(ADP-ribosyl)ated PARP1 (Fig. 4b, lane *12*), because automodification decreases its electrophoretic mobility [33]. The modified enzyme was likely to incompletely lose the ability to bind DNA, and this explained the visualization of high-molecular-mass crosslinked products of the PARP1 in the presence of NAD^+. Analysis of these products after their treatment with benzonase showed a band corresponding in the mobility to unmodified PARP1 (Fig. 4b, compare lanes *10* and *11*, *12*), and benzonase seemed to remove poly(ADP-ribose).

DISCUSSION

At present, the main pathways of BER are reconstituted *in vitro* and the enzymology of this process is well studied [2-4, 26]. However, recent studies suggest that the *in vivo* mechanism of BER is more complicated, as additional proteins (enzymes) influencing this process have been discovered [5, 10]. Thus, the question of the functional involvement and influence of PARP1 on BER is still open. In particular, the interaction of PARP1 with DNA breaks is supposed to prevent functioning of the repair enzymes [7]. In this case, the interaction of PARP1 with DNA is regulated through its autopoly(ADP-ribosyl)ation that is initiated on the interaction of PARP1 with DNA breaks. The covalent attachment of poly(ATP-ribose) to PARP1 is supposed to result in dissociation of the PARP1–DNA complex and thus provide access of repair enzymes to damaged DNA [7, 34]. But, according to the alternative model, PARP1 recognizes and temporarily protects the DNA ends in the point of the break and then promotes formation of the BER complex [35, 36]. Generation of protein–protein interactions between PARP1 and BER proteins such as pol β, XRCC1, and DNA ligase III has been demonstrated by co-immunoprecipitation and yeast two-hybrid analysis [37]. Generation of $PARP1^{-/-}$ cell lines promoted comparison of efficiencies of DNA repair in cell extracts from wild

type and PARP1$^{-/-}$ mouse fibroblasts [6]. The results were contradictory: PARP1 was shown to stimulate, suppress, and have no effect on the recovery of DNA structure for different types of damage [38-41].

The effect of PARP1 on BER in the system reconstituted with purified proteins is also ambiguous and depends on the ratio of concentrations of DNA, repair proteins, and PARP1. Thus, PARP1 itself in DNA-comparable concentration failed to influence the efficiency of DNA synthesis catalyzed by pol β, whereas the DNA synthesis by the long-patch subpathway of BER was stimulated by PARP1 when present with FEN1 [42]. However, under excess concentration of PARP1 relatively to DNA, the efficiency of one nucleotide gap filling by pol β was decreased and the strand-displacement DNA synthesis was inhibited [18, 43]. Notwithstanding contradictions of *in vitro* data on stimulation or suppression of separate stages of BER by PARP1, studies on the PARP1 deficient cells and organism clearly indicated the necessity of PARP1 involvement in the cell response to genotoxic stress. This is especially important in the case of massive damage of DNA bases leading to apoptotic or necrotic cell death [6, 9, 10]. During apoptosis, PARP1 is inactivated by enzymatic proteolysis with production of the 24-kD fragment capable of DNA binding. In contrast to PARP1, the interaction of p24 with DNA cannot be regulated through poly(ADP-ribosyl)ation [11]. It is supposed that, on interaction with DNA breaks, p24 can inhibit the repair by sterically preventing interaction of the repair factors with damaged DNA [14, 15].

We have compared the influence of exogenous PARP1 and its apoptotic 24-kD fragment on the repair in the nuclear extract of DNA duplexes imitating substrates of the long-patch and short-patch subpathways of BER (DNA-1 and DNA-2, respectively). On the repair of both substrates, exogenous PARP1 or p24 decreased the efficiency of gap filling DNA synthesis and suppressed the strand-displacement synthesis. The effective incorporation of ddCMP observed at the gap filling suggested that pol β could catalyze DNA synthesis during this stage of DNA-1 and DNA-2 repair, and the dominating of the "gap translation" mechanism also allowed us to consider pol β to be the major polymerase catalyzing DNA synthesis during the repair of DNA-1 by the long-patch subpathway. It was earlier shown by photosensitized modification that in the nuclear extract mainly pol β interacted with DNA imitating a long-patch BER intermediate [17]. Therefore, the decreased efficiency of this stage of the repair in the presence of exogenous PARP1 or p24 can be considered as a consequence of the competition between these proteins and pol β of the extract on the interaction with DNA substrate. The gap filling leads to generation of DNA intermediates containing a nick with phosphate (for DNA-2) or furan phosphate (for DNA-1) at the 5′-end of the downstream primer. During the repair of DNA-2, the nick is ligated rather effectively even in the presence of exogenous PARP1 and p24. It was suggested that DNA ligase III catalyzed the joining of a nick during short-patch subpathway of BER [2, 6]. As opposed to other eukaryotic ligases, DNA ligase III contains the DNA-binding motif "zinc fingers" homologous to "zinc fingers II" (F II) of PARP1 and responsible for the interaction with single-strand DNA breaks [37]. This motif is supposed to be necessary for the ligase functioning in the presence of PARP1 [37, 44].

The addition of PARP1 or p24 suppressed the forming of intact structure of DNA-1. In the presence of PARP1 or p24, the strand-displacement DNA synthesis was inhibited, as well as the endonuclease activity of FEN1 of the nuclear extract.

The kinetic analysis has shown that on the repair of DNA-1, DNA synthesis is mainly passed by the "gap translation" mechanism, when during formation of the nick-containing DNA with the flapping furan phosphate at the 5′-end of the downstream primer FEN1 creates a new gap through removal of nucleotide with 5′-furan phosphate. Then pol β incorporates dNMP, and a nick is generated with hydroxyl group at the 3′-end and phosphate at the 5′-end ready to be ligated. In this case, when one nucleotide gapped DNA is created *de novo* DNA synthesis has to occur in the extract even in the presence of exogenous PARP1 and p24. Moreover, we suppose that during the "gap translation" mechanism the nick sealing may be catalyzed by DNA ligase III and not DNA ligase I, because [^{32}P]AMP-intermediate of DNA ligase III is used for the repair of DNA-1. In this case, the intact structure of DNA-1 can be also formed in the presence of exogenous PARP1 and p24, as shown for DNA-2. According to the literature, pol δ can mediate long patch DNA synthesis, and then resynthesis of DNA results in a flap structure, i.e., DNA intermediate with the flapping 5′-end [45]. PARP1 fails to directly affect the activity of pol δ but can lower the efficiency of DNA synthesis by binding its processivity factor PCNA [45, 46].

Note that the 24-kD fragment is unable to inhibit the activity of pol δ by this mechanism, because PARP1 interact with PCNA through its NAD^+-binding domain. Thus, PARP1 and the 24-kD fragment decrease the efficiency of DNA synthesis on the repair of DNA-1 but do not completely suppress this stage of the repair. Both mechanisms of long-patch DNA synthesis, the "gap translation" or strand-displacement synthesis, are realized with involvement of FEN1 for forming of either a one nucleotide gap for pol β or a nick with 5′-phosphate and 3′-hydroxyl group for DNA ligase action [4, 30]. Therefore, the suppressed formation of the intact structure of DNA duplex during the repair of DNA-1 in the presence of exogenous PARP1 and p24 can be mainly caused by inhibition of the FEN1 activity. This hypothesis is supported by our data on the decrease in the efficiency of endonuclease cleavage catalyzed by both the extract proteins and recombinant FEN1 in the presence

of these proteins. The results of photoaffinity modification of the nuclear extract proteins confirm our data of functional analysis. PARP1 and its 24-kD fragment effectively compete with the extract proteins on the interaction with nicked DNA BER intermediates.

The nicked photoreactive DNA intermediate of the short-patch pathway was ligated in the presence of ATP, and, as a consequence, the labeling level of exogenous proteins decreased, and addition of PARP1 or its 24-kD fragment had no effect on this process. ATP did not influence the modification of PARP1 and p24 by the photoreactive DNA intermediate of the long-patch subpathway of BER, and the addition of NAD^+ insignificantly decreased the level of exogenous PARP1 labeling, which on the photomodification gave products corresponding to its poly(ADP-ribosyl)ated form. And the incomplete recovery of the DNA-synthesizing and endonuclease activities of the extract enzymes responsible for cleavage of the 5′-end of furan phosphate-containing oligonucleotide in the presence of exogenous PARP1 and NAD^+ also suggested a possibility of interaction between the automodified PARP1 and DNA substrate.

Thus, the interaction of PARP1 and p24 with DNA intermediates of the long-patch BER subpathway resulted in suppression of the subsequent stages of the repair following by this mechanism. However, the DNA duplex is rather effectively repaired by the short-patch subpathway in the presence of exogenous PARP1 and p24. Moreover, analysis of the repair in the nuclear extract of DNA duplexes and also results of photoaffinity modification have shown that the same BER extract enzymes can catalyzed separate stages of the long patch and short patch subpathways of BER, and this suggests a similar mechanism of coordination of these processes in the cell.

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