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Affinity Labeling of Flap-endonuclease FEN-1 by Photoreactive DNAs

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Abstract—Eukaryotic flap-endonuclease (FEN-1) is 42-kD single-subunit structure-specific nuclease that cleaves 5′-flap strands of the branched DNA structure and possesses 5′-exonuclease activity. FEN-1 participates in DNA replication, repair, and recombination. The interaction of FEN-1 with DNA structures generated during replication and repair was studied using two types of photoreactive oligonucleotides. Oligonucleotides bearing a photoreactive arylazido group at the 3′-end of the primer were synthesized *in situ* by the action of DNA polymerase β using base-substituted photoreactive dUTP analogs as the substrates. The photoreactive group was also bound to the 5′-end phosphate group of the oligonucleotide by chemical synthesis. Interaction of FEN-1 with both 5′- and 3′-ends of the nick or with primer–template systems containing 5′- or 3′-protruding DNA strands was shown. Formation of a structure with the 5′-flap containing the photoreactive group results in decrease of the level of protein labeling caused by cleavage of the photoreactive group due to FEN-1 endonuclease activity. Photoaffinity labeling of proteins of mouse fibroblast cell extract was performed using the radioactively labeled DNA duplex with the photoreactive group at the 3′-end and the apurine/apyrimidine site at the 5′-end of the nick. This structure is a photoreactive analog of an intermediate formed during DNA repair and was generated by the action of cell enzymes from the initial DNA duplex containing the 3-hydroxy-2-hydroxymethyltetrahydrofurane residue. FEN-1 is shown to be one of the photolabeled proteins; this indicates possible participation of this enzyme in base excision repair.

Key words: photoaffinity labeling, protein–nucleic acid interaction, flap-endonuclease, DNA repair

The flap 5′-end DNA structures resulting from displacement of a down-stream primer during replication of the complementary strand by DNA polymerase are important intermediates in many reactions of DNA metabolism. Formation and subsequent excision of such structure allow removal of certain types of DNA damage. This mechanism operates in case of base excision repair [1]. It was also supposed that formation of flap structures occurs in nucleotide repair [2-4], homologous recombination [5, 6], and during processing of Okasaki fragments [7-11]. Flap-endonuclease (FEN-1) plays a key role in cleavage of such structures [12]. FEN-1 cleaves the 5′-flap strand of DNA and possesses 5′-exonuclease activity [12]. Specificity of this enzyme against various DNA structures was studied by gel retardation [13]. Photoaffinity labeling can be an alternative approach to the study of FEN-1–DNA interaction. We developed a method of photoaffinity labeling using partial DNA duplexes containing photoreactive groups at the 3′-end of the primer, incorporated by DNA polymerases [14, 15]. Photoreactive groups can be incorporated into the 5′-end of DNA by chemical synthesis [16]. Affinity labeling is of particular interest for the study of FEN-1–DNA interaction, because it allows to study this interaction in reconstructed systems as well as in the cell and nuclear extracts.

Abbreviations: FAP-dUTP) 5-{N-[N-(4-azido-2,5-difluoro-3-chlorpyridine-6-yl)-3-aminopropionyl]-*trans*-3-aminopropen-1-yl}-2′-deoxyuridine-5′-triphosphate; FAB-dUTP) 5-[N-(2,3,5,6-tetrafluoro-4-azidobenzoyl)-*trans*-3-aminopropen-1-yl]-2′-deoxyuridine-5′-triphosphate; R) 2-nitro-5-azidobenzoyl group bound to the 5′-end phosphate group of oligonucleotide (see Fig. 1); Pol β) DNA polymerase β; BSA) bovine serum albumin; FEN-1) flap-endonuclease; AP-endonuclease) apurine/apyrimidine endonuclease.

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In this work, we are the first to present data on photoaffinity labeling of FEN-1 by the DNA duplex bearing a photoreactive group at the 3′-end and the DNA duplex bearing a photoreactive group at the 5′-end phosphate. The results are promising for the study of FEN-1 substrate specificity and FEN-1–DNA interaction in cell and nuclear extracts. In the present work the photoaffinity labeling of FEN-1 of the mouse fibroblast cell extract was performed using photoreactive DNA analogous to a repair intermediate formed by the action of cellular DNA repair enzymes.

MATERIALS AND METHODS

The following reagents were used in this study: [γ-^{32}P]ATP and [α-^{32}P]dCTP from Biosan (Russia); T_4 polynucleotide kinase from Sibenzim (Russia); dATP, dGTP, TTP, Sigmamarker High Range molecular mass markers, reagents for electrophoresis, and main components of buffers from Sigma (USA). Other reagents were of special purity and chemical grade and of Russian production.

Recombinant rat Pol β was isolated from *Escherichia coli* according to a standard procedure [17] but with some modifications. Recombinant FEN-1 and AP-endonuclease were kindly donated by Dr. P. Prasad (National Institute of Environmental Health, North Carolina, USA). Mouse fibroblast cell extract was obtained according to [18].

Synthesis and photochemical properties of FAP-dUTP, and FAB-dUTP were described earlier [15, 19].

DNA duplexes were constructed using the following oligonucleotides (Genset, USA): 42-mer 5′-GGTTCGATATCGTAGTTCTATTATTTATGTATAGCCCCTACC (I), 22-mer 5′-GGTAGGGGCTATACATAAATAA (II), 17-mer 5′-p-AGAACTACGATATCGAA (III) and AGAACTACGATATCGAA (IV), and also 34-mer 5′-CTGCAGCTGATGCGCFGTACGGATCCCCGGGTAC (V) containing the 3-hydroxy-2-hydroxymethyltetrahydrofurane (F) residue and complementary to it 5′-GTACCCGGGGATCCGTACAGCGCATCAGCTGCAG (VI) (the structures of duplexes are presented in Fig. 1).

Binding of the photoreactive nitroazidobenzoyl group to the 5′-end phosphate group of oligonucleotide (III) was performed as described earlier [16]; the resulting photoreactive oligonucleotide (IIIa) was used as a photoaffinity reagent.

Radioactive label was incorporated into the 5′-end of oligonucleotide as described earlier [20]. Labeled oligonucleotides were purified by PAGE with subsequent elution [21].

The radioactively labeled primer (II) was elongated with Pol β in 10 μl of the reaction mixture containing 50 mM Tris-HCl, pH 7.5 (25°C), 50 mM NaCl, 5 mM $MgCl_2$, 0.5 μM duplex, 0.4 μM Pol β, 1 μM FAP-dUTP, and natural dNTP (in accord with the template structure) in case of primer elongation by more than one step. The mixtures were incubated for 20 min at 37°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/bis-acrylamide = 20 : 1) in the presence of 7 M urea; the gels were 0.4 mm in thickness and 300 mm in length. Electrophoresis was performed in 100 mM Tris-borate buffer, pH 8.3, at 1000 V.

Oligonucleotide (IIIa) bearing a photoreactive group at the 5′-end phosphate was radioactively labeled by Pol β in the presence of [α-^{32}P]dCTP in 10 μl of reaction mixture. The reaction conditions are described above.

Photoaffinity labeling of Pol β and FEN-1 by photoactive oligonucleotides synthesized *in situ* was performed in 15 μl of the reaction mixture containing 0.4 μM Pol β, 0.5 μM primerized or nick-containing duplex, and 1 μM FAP-dUTP in the standard buffer. Incorporation of the photoreactive residue FAP-dUMP to the 3′-end of the 5′-^{32}P-labeled primer (II) was performed as described above. Then FEN-1 was added to the final concentrations 0.8, 0.6, or 0.4 μM and the resulting mixtures were UV-irradiated.

Photoaffinity labeling of Pol β and FEN-1 by oligonucleotide (IIIa) bearing a photoreactive group at the 5′-end was performed in 15 μl of reaction mixture containing 0.4 μM Pol β, 0.4 μM primed or nick-containing duplex, and 1 μM [α-^{32}P]dCTP in the standard buffer. Incorporation of [^{32}P]dCMP to the 3′-end of oligonucleotide (IIIa) was performed as described above. Then FEN-1 was added to the final concentrations 0.8, 0.6, or 0.4 μM and the resulting mixtures were UV-irradiated.

Photoaffinity labeling of proteins of the mouse fibroblast cell extract was performed in 15 μl of the reaction mixture containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.2 μM ^{32}P-labeled F-DNA, 10 μM FAB-dUTP, and proteins of the cell extract at the total concentration 1 mg/ml. The reaction mixture was incubated for 30 min at 25°C and then UV-irradiated under the standard conditions. For the reconstructed system, 1 μM AP-endonuclease and Pol β and 0.46 μM FEN-1 were used.

UV-irradiation was performed using a DRK-120 high-pressure mercury lamp; the VIO-1 condenser was from LOMO (St. Petersburg, Russia). The distance was 110 mm through the light filter WG-280 (>280 nm), $I = 1.2 \cdot 10^{15}$ quanta/sec on 1 cm^2. The products of modification were analyzed by SDS-PAGE [22] with subsequent autoradiography.

RESULTS AND DISCUSSION

Structures of oligonucleotide duplexes used in this study are presented in Fig. 1. It is known that on filling

a
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5'-GGTTCGATATCGTAGTTCTATTATTTATGTATAGCCCCTACC-3'   I
                          3'-AATAAATACATATCGGGGATGG-5'   II
```

b
```
5'-GGTTCGATATCGTAGTTCTATTATTTATGTATAGCCCCTACC-3'   I
  3'-AAGCTATAGCATCAAGA-p-5'                        III
                          3'-AATAAATACATATCGGGGATGG-5'   II
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c
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5'-GGTTCGATATCGTAGTTCTATTATTTATGTATAGCCCCTACC-3'   I
  3'-AAGCTATAGCATCAAGA-5'                          IV
                          3'-AATAAATACATATCGGGGATGG-5'   II
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d
```
5'-GGTTCGATATCGTAGTTCTATTATTTATGTATAGCCCCTACC-3'   I
  3'-AAGCTATAGCATCAAGA-R-5'                        IIIa
```

e
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5'-GGTTCGATATCGTAGTTCTATTATTTATGTATAGCCCCTACC-3'   I
  3'-AAGCTATAGCATCAAGA-R-5'                        IIIa
                          3'-AATAAATACATATCGGGGATGG-5'   II
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f
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5'-CTGCAGCTGATGCGCFGTACGGATCCCCGGGTAC-3'           V
3'-GACGTCGACTACGCGACATGCCTAGGGGCCCATG-5'           VI
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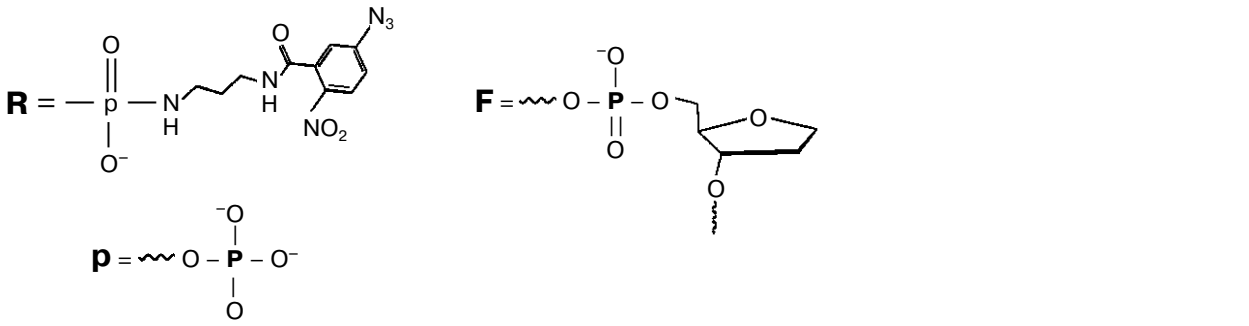

Fig. 1. Structures of oligonucleotide duplexes: with 5′-protruding end of template (a); containing mononucleotide gap with nonphosphorylated (b) and phosphorylated (c) down-stream primers; containing primers with photoreactive 5′-ends (d and e); containing 3-hydroxy-2-hydroxymethyltetrahydrofurane (f).

single-stranded gaps Pol β is able to synthesize DNA with displacement of the down-stream primer; this results in formation of a 5′-flap structure that is a substrate for FEN-1 [23, 24]. In the first step, it was demonstrated that synthesis with displacement of the down-stream primer using a photoreactive dNTP analog as the substrate is possible, and the effect of the 5′-end phosphate group of the down-stream oligonucleotide on efficiency of the synthesis was also studied. The results of electrophoretic separation of the products of reaction of the primer elongation in structure (a) (lanes *1-5*) and in duplexes having a mononucleotide gap with the 5′-phosphorylated (lanes *6-10*) and nonphosphorylated (lanes *11-15*) down-stream primer (structures (b) and (c), respectively) in the presence of FAP-dUTP and natural substrates dATP, dGTP, and dCTP are presented in Fig. 2. As shown, elongation of the 5′-^{32}P-labeled primer with Pol β by 1, 2, and 4 units is observed in the duplex with 5′-protruding end of the template (lanes *2-4*) as well as in structures with the gap (lanes *7-9*, *12-14*). It should be noted that the presence of the 5′-end phosphate inside the gap did not influence the primer elongation to 4 units (lanes *7-9*). However, con-

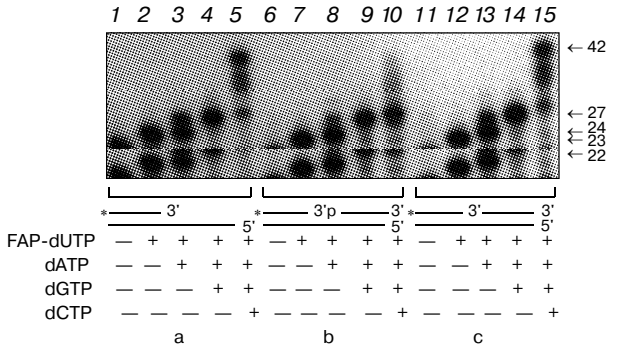

Fig. 2. Products of primer elongation in the structure with 5′-protruding end of the template (a) and in mononucleotide gaps (b, c). Lanes: *1*, *6*, *11*) initial primer; *2*, *7*, *12*) primer elongation by one unit; *3*, *8*, *13*) primer elongation by two units; *4*, *9*, *14*) primer elongation by four units; *5*, *10*, *15*) complete primer elongation. From here on ^{32}P-containing groups are marked by asterisks. Lengths of the initial primer and the products of its elongation in nucleotide residues are given at the right.

trary to structures (a) and (c) (lanes *5* and *15*, respectively), in the case of structure (b) complete elongation of the primer did not occur (*10*). This result seems to demonstrate participation of the 5′-end phosphate group in regulation of the length of Pol β-catalyzed synthesis with displacement of the down-stream primer flanking the gaps [25]. It is possibly the interaction of Pol β with the 5′-end phosphate that limits its activity in DNA synthesis with displacement of the blocking primer and makes participation of other DNA polymerases in filling large gaps necessary [26].

Since FEN-1 is a structure-specific nuclease and does not exhibit any enzymatic activity toward the 3′-ends of DNA, the possibility of modifying this protein using oligonucleotides with a photoreactive group at the 3′-end was not obvious. Data on FEN-1 modification by a photoreactive primer in the complex of duplex (a) bearing at the 3′-end a FAP-dUMP residue incorporated by Pol β compared to modification by oligonucleotide (IIIa) in the composition of duplex (d) bearing a photoreactive group chemically bound to the 5′-end phosphate are presented in Fig. 3. A radioactive label was incorporated into the photoreactive oligonucleotide (IIIa) by Pol β in the presence of [α-^{32}P]dCTP. Two [^{32}P]dCMP residues were incorporated into the 3′-end of the primer to fit the template structure (data not presented here). As shown in Fig. 3, modification of FEN-1 is observed in both cases, the intensities of FEN-1 labeling by 3′-end and 5′-end reagents being comparable (lanes *3-5* and *8-10*, respectively). However, comparing the labeling efficiencies of Pol β and FEN-1, it can be noted that, in the presence of FEN-1, Pol β modification by the 5′-end reagent (lanes *8-10*) is significantly smaller than by the 3′-end reagent (lanes *3-5*); this indicates a stronger interaction of FEN-1 with the 5′-end of DNA.

Interaction of repair proteins as a whole and flap-endonuclease in particular with specific DNA structures forming during a repair process, such as a nick or a flap,

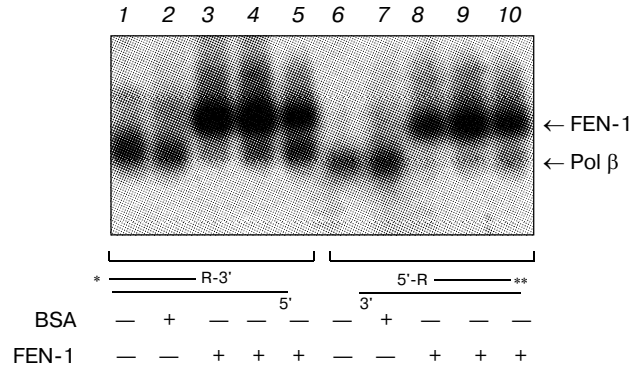

Fig. 3. Photoaffinity labeling of DNA polymerase β and FEN-1 by photoreactive oligonucleotides in the presence of the (a) and (d) duplexes. Labeling of DNA polymerase β without FEN-1 (lane *1*) and with addition of BSA (lane *2*) and FEN-1 at concentrations 0.8 (*3*), 0.6 (*4*), and 0.4 μM (*5*) by photoreactive primer bearing a FAP-dUMP residue at the 3′-end. Labeling of DNA polymerase β in the absence of FEN-1 (lane *6*) and with addition of BSA (lane *7*) and FEN-1 at concentrations 0.8 (*8*), 0.6 (*9*), and 0.4 μM (*10*) by photoreactive primer with photoreactive 5′-end.

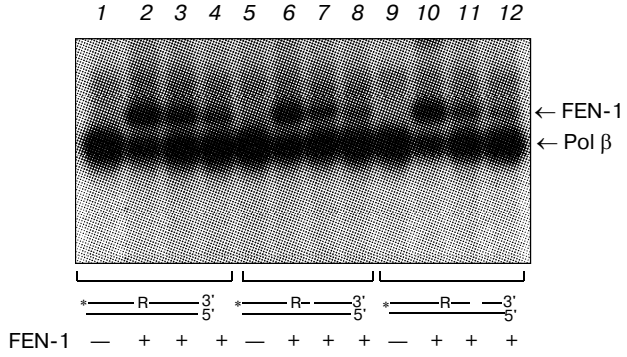

Fig. 4. Photoaffinity labeling of DNA polymerase β and FEN-1 by photoreactive oligonucleotides in the presence of duplex (b). Labeling of DNA polymerase β without FEN-1 (lane *1*) and with FEN-1 at concentrations 0.8 (*2*), 0.6 (*3*), and 0.4 μM (*4*) by photoreactive primer bearing a FAP-dUMP residue at the 3′-end. Labeling of DNA polymerase β in the absence of FEN-1 (lane *5*) and presence of FEN-1 at concentrations 0.8 (*6*), 0.6 (*7*), and 0.4 μM (*8*) by the primer bearing a photoreactive group penultimate position from the 3′-end of the nick. Labeling of DNA polymerase β in the absence of FEN-1 (lane *9*) and presence of FEN-1 at concentrations 0.8 (*10*), 0.6 (*11*), and 0.4 μM (*12*) by the primer bearing a photoreactive group four nucleotide residues apart from the 3′-end of the nick.

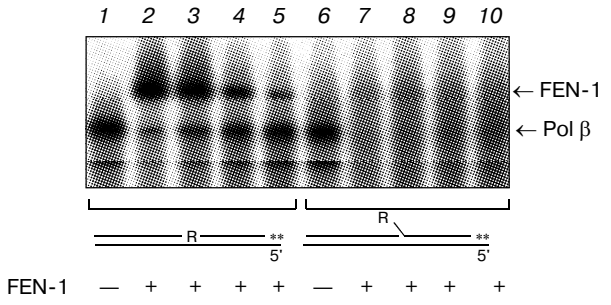

Fig. 5. Photoaffinity labeling of DNA polymerase β and FEN-1 by photoreactive oligonucleotides in the presence of duplex (e). Labeling of DNA polymerase β in the absence of FEN-1 (lane *1*) and presence of FEN-1 at concentrations 0.8 (*2*), 0.6 (*3*), 0.4 (*4*), and 0.2 μM (*5*) by photoreactive primer with a photoreactive 5′-end inside the nick. Labeling of DNA polymerase β in the absence of FEN-1 (lane *6*) and presence of FEN-1 at concentrations 0.8 (*7*), 0.6 (*8*), 0.4 (*9*), and 0.2 μM (*10*) by photoreactive primer with a photoreactive 5′-flap strand.

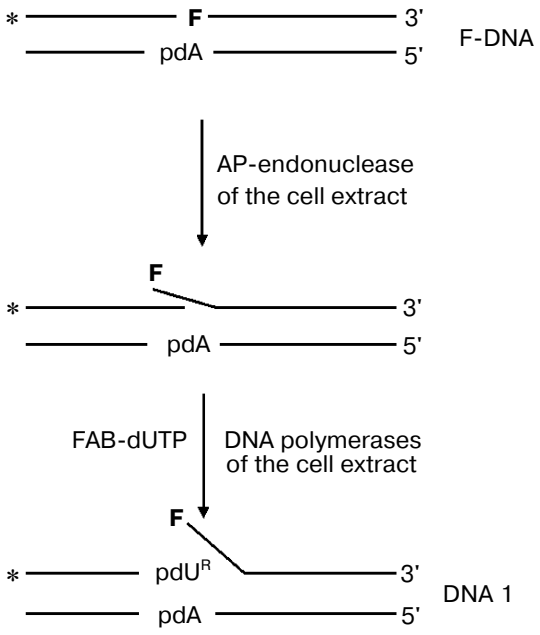

Fig. 6. Scheme of incorporation of photoreactive analog into oligonucleotide duplex modeling a repair intermediate.

is of special interest. We studied the interactions of Pol β and FEN-1 with 3′- and 5′-ends of DNA forming a nick and the possibility to use 5′-flap DNA for photoaffinity labeling of proteins in the presence of FEN-1. Structures (b) and (e) are converted into the structures with photoreactive groups at the 3′- and 5′-end of the nick after Pol β-catalyzed gap filling by the FAP-dUMP (structure (b)) or TMP (structure (e)) residues, respectively, were used as the initial substrates. Data on Pol β and FEN-1 modification by oligonucleotides with a photoreactive group at the 3′-end or one or four nucleotide residues apart from it are presented in Fig. 4. As shown above, Pol β is able to perform a synthesis with displacement of the blocking primer. Addition of natural dATP along with FAP-dUTP to the reaction mixture results in moving the photoreactive group aside the 3′-end of the primer by one nucleotide residue; addition of the structurally next dGTP results in moving aside by four residues. Thus, a flap structure that is to be cleaved by FEN-1 is formed at the 5′-end of the down-stream primer. So, protein modification by a photoreagent localized at the 3′-end of the nick or near it is observed. As shown in Fig. 4, moving of the photoreactive group aside the 3′-end of the primer by one (lanes *5-8*) or four (lanes *9-12*) nucleotide units results in decrease in labeling intensity of both FEN-1 and Pol β compared to modification by oligonucleotide with a photoreactive group just at the 3′-end (lanes *1-4*).

Data on the modification of FEN-1 and Pol β by oligonucleotide with a photoreactive 5′-end either inside the nick (lanes *1-5*) or forming a flap structure (lanes *6-10*) are presented in Fig. 5. As shown, FEN-1 labeling by the 5′-end reagent occurs only in the nick (lanes *2-5*). In case the group is located at the 5′-flap strand, products of

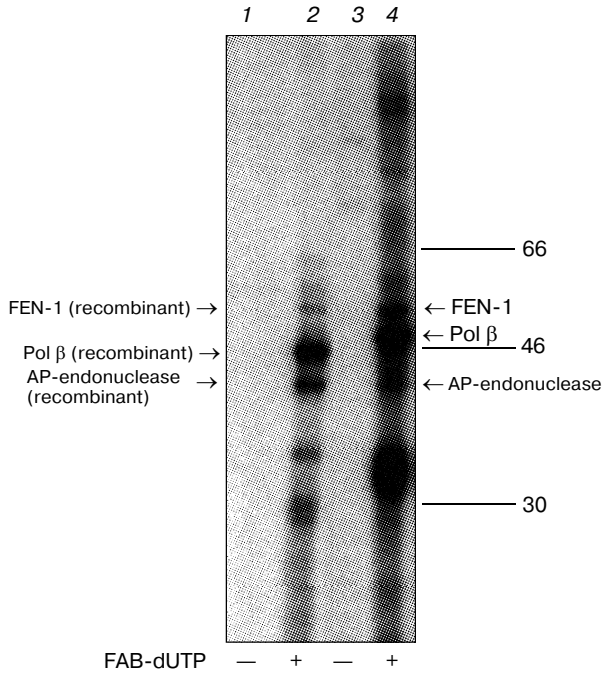

Fig. 7. Photoaffinity labeling of DNA polymerase β, FEN-1, and AP-endonuclease by photoreactive DNA 1 (see Fig. 6): in the reconstructed system (lane *2*), in the mouse fibroblast cell extract (lane *4*), in the absence of FAB-dUTP in the reconstructed system and in the mouse fibroblast cell extract (lanes *1* and *3*, respectively). Molecular masses of protein markers are given on the right (kD): 66 (BSA), 46 (ovalbumin), and 30 (carbonic anhydrase).

photo-cross-linking with FEN-1 as well as with Pol β are not observed. This can be explained by the fact that FEN-1 cleaves the flap structure with a photoreagent at the 5′-end, thus drastically decreasing labeling. Pol β labeling is observed in the absence of FEN-1 (lane *6*), and this counts in favor of our suggestion. So, the results indicate that photoreactive structures with a photoreactive group at both the 3′-end and the 5′-end can be used for studies of the repair system. However, DNAs bearing a photoreactive group at the 3′-end of the nick are more promising for analysis of the complete repair complex at the cell extracts because of the activity of FEN-1 cleaving 5′-flap structures.

We used photoaffinity labeling of FEN-1 by the 3′-end reagent in the nick for identifying this protein in the mouse fibroblast cell extract. Oligonucleotide duplex (f) was used as the initial substrate (Fig. 1). This substrate is a good model for the study of repair process [27, 28] because it contains a 3-hydroxy-2-hydroxymethyltetrahydrofurane (F) residue modeling an apyrimidine/apurine site, which is not excised by Pol β lyase activity [29, 30]. That is why this structure allows formation of the nick bearing the photoreactive dNMP residue at the 3′-end and the apurine/apyrimidine site at the 5′-end. This structure can be considered as a photoreactive analog of the DNA repair intermediate [26, 27, 31]. Oligonucleotide duplex (f) containing 5′-^{32}P-labeled oligonucleotide V (F-strain) was incubated with the cell extract in the presence of FAB-dUTP under the conditions described in "Materials and Methods". A photoreactive intermediate bearing the FAB-dUMP residue at the 3′-end of the nick was formed by the action of cell enzymes according to a scheme shown in Fig. 6. In accord with the duplex structure, only one FAB-dUMP residue was incorporated into the elongated 3′-end of the primer (data not shown). Subsequent UV-irradiation of the photoreactive duplex (DNA 1 in Fig. 6) in the presence of the cell extract resulted in photoaffinity labeling of a limited number of proteins (Fig. 7). The modified proteins were identified as DNA polymerase β, FEN-1, and AP-endonuclease by immunoprecipitation using antibodies specific to repair proteins (results not shown). To confirm the identity of the products of photo-cross-linking to the above-mentioned enzymes, analogous experiments with an enzymatic system reconstructed from recombinant DNA polymerase β, flap-endonuclease, and AP-endonuclease were performed, the results being presented in Fig. 7. As shown, electrophoretic mobility of the products of photolabeling of FEN-1 and AP-endonuclease in the reconstructed system (lane *2*) coincides with that of analogous products in the cell extract (*4*), whereas mobilities of recombinant (*2*) and cellular (*4*) Pol β differ. It should be noted that in the absence of FAB-dUTP photo-cross-linking occurs neither in the reconstructed system nor in the cell extract (Fig. 7, lanes *1* and *3*, respectively).

Thus, we have demonstrated that FEN-1 efficiently interacts with photoreactive structures modeling repair intermediates. Photoaffinity labeling of the mouse fibroblast cell extract proteins was performed by a DNA repair intermediate bearing a photoreactive group at the 3′-end and the apurine/apyrimidine site at the 5′-end. FEN-1 was proved to be one of the modified proteins; this indicates the usefulness of this approach for identification of this protein and other constituents of the repair complex on the cell extract level.

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