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Use of Modified Flap Structures for Study of Base Excision Repair Proteins

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Abstract—To investigate interactions between proteins participating in the long-patch pathway of base excision repair (BER), DNA duplexes with flap strand containing modifications in sugar phosphate backbone within the flap-forming oligonucleotides were designed. When the flap-forming oligonucleotide consisted of two sequences bridged by a decanediol linker located in the flap strand near the branch point, the efficiency and position of cleavage by flap endonuclease 1 (FEN1) differed from those for natural flap. The cleavage rate of chimeric structure by FEN1 was lower than that of a normal substrate. When we introduced the second modification in the flap-forming oligonucleotide, the cleavage rate decreased significantly. To estimate efficiency of recognition and processing of the chimeric structures by BER proteins, we studied the rate of DNA synthesis by DNA polymerase β (Pol β) and the rate of nucleotide excision at the 3′-end of the initiating primer by apurinic/apyrimidinic endonuclease 1 (APE1) compared with those for the natural DNA duplexes. Efficiency of strand-displacement DNA synthesis catalyzed by Pol β was shown to be higher for flap structures containing non-nucleotide linkers. The chimeric structures were processed by the 3′-exonuclease activity of APE1 with efficiency lower than that for a normal flap structure. Thus, DNA duplexes with modifications in sugar phosphate backbone can be used to mimic intermediates of the long-patch pathway of BER in reconstituted systems containing FEN1. Based on chimeric and natural oligonucleotides, photoreactive DNA structures were designed. The photoreactive dCMP moiety was introduced into the 3′-end of DNA primer via the activity of Pol β. The photoreactive DNA duplexes—3′-recessed DNA, nicked DNA, and flap structures containing natural and chimeric oligonucleotides—were used for photoaffinity labeling of BER proteins.

Key words: flap endonuclease 1, base excision repair, photoaffinity labeling

In cells, DNA is constantly subjected to damage by exogenous and endogenous agents. There are several systems of DNA repair, which specialize in removing damages of a certain type. Base excision repair (BER) is one of the main types of repair. So far, two pathways of BER differing in the number of incorporated nucleotides and the set of proteins participating in repair are known. The first pathway is short-patch or mononucleotide excision repair; it includes subsequent action of several proteins. DNA glycosylase excises a damaged base. Then apurinic/apyrimidinic endonuclease cleaves the DNA sugar phosphate backbone at the 5′-position in relation to the apurinic/apyrimidinic site, forming a nick flanked by the hydroxy group at the 3′-end and deoxyribose phosphate (dRP) at the 5′-end. Further action of DNA polymerase β (Pol β) results in inclusion of one dNMP residue by polymerase activity and excision of dRP residue by the lyase function [1, 2]. dRPase activity of Pol β has been shown to be lower than its polymerase activity [3]. The lyase activity of Pol β is sensitive to the structure of sugar phosphate moiety. Chemically modified (for example, oxidized or reduced) deoxyribose residues are refractory to the lyase activity. As a result, repair switches to the long-patch pathway with inclusion of several (from 2 to 10) nucleotides. This type of repair is performed with participation of other proteins—replicative DNA polymerases δ and ε and proliferating cell nuclear antigen (PCNA) [4, 5]. These polymerases catalyze strand-displacement DNA synthesis resulting in formation of a flap strand. Pol β can also catalyze strand-displacement DNA synthesis with the same result [5, 6]. Flap endonuclease 1 (FEN1) is a key enzyme for processing structures of this type. FEN1 belongs to endonu-

Abbreviations: BER) base excision repair; APE1) human apurinic/apyrimidinic endonuclease 1; dRP) deoxyribose phosphate residue; FEN1) human flap endonuclease 1; Pol β) human DNA polymerase β; PARP1) human poly(ADP-ribose) polymerase 1; XRCC1) X-ray repair cross-complementing group 1 protein; AP site) apurinic/apyrimidinic site; PNK) T4 phage polynucleotide kinase.

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cleases strictly specific to the substrate structure. Flap endonuclease is active in the presence of Mg^{2+} [7]. 5′-Flap structures are natural substrates for this enzyme. The former is a complex consisting of three DNA strands, one of which having unpaired 5′-flap strand. Such structures can also be formed during DNA recombination and processing of the Okasaki fragments in DNA replication. FEN1 recognizes 5′-flap strand of the substrate and moves along it to the excision sites located at the penultimate position from the branch point and/or one nucleotide after it [7]. If double-strand sites or large-sized adducts are within the flap, the nuclease activity of the enzyme is inhibited [8]. Activity also decreases in the presence of proteins that can bind to the single-strand DNA. In this case, FEN1 and DNA-binding protein compete for the substrate. We showed [9] that inhibition of FEN1 by replication protein A depends on the dimension of the flap strand of the substrate. When the flap strand was 20 nucleotides in length (enough for efficient binding of replication protein A (RPA)), cleavage was substantially inhibited, whereas for structures with a short flap strand cleavage efficiency did not depend on the presence of RPA. Inhibition of FEN1 by RPA in case of structures with a long flap was also noted in [10]. The data indicate that FEN1 slides along all the unpaired DNA strand to the branch point, where DNA is cleaved. Sliding is possible in two directions: towards and away from the branch point. When using primers about 20 nucleotides in length complementary to the 5′-end or to the middle part of a flap 73 nucleotides in length, a minor degree of cleavage was observed only in the case of FEN1 preincubation with the substrate before addition of the primers. When FEN1 is added to the mixture already containing oligonucleotide complementary to the flap strand, the enzyme is not bound. Addition of complementary primers after preincubation of the enzyme with substrate results in more efficient complex formation than in the absence of primers. FEN1 appears to be "locked" at the site between the complementary oligonucleotide and the branch point and thus cannot dissociate. This fact indicates that the enzyme can move along the single-strand DNA in both directions. When using oligonucleotide complementary to the site near the branch point, the products of cleavage are not formed even after FEN1 preincubation with DNA. In spite of the presence of free single-strand site enough for FEN1 location, formation of a protein–DNA complex was not observed by gel retardation assay. This indicates that the tightest FEN1 binding to the substrate is in the branch point [8].

Photoaffinity modification is a widely used approach when interactions between components of the complexes of DNA replication and repair are studied in systems reconstructed from the purified proteins as well as on the cell extract level. Photoaffinity modification can reveal new proteins specifically interacting with DNA structures, which mimic intermediates of the repair process. However, cell extract proteins can process DNA intermediates, forming structures of other types, and thus it is important to know the DNA type we deal with. Since it is difficult to use the photoreactive flap structures in extracts due to their cleavage in the presence of Mg^{2+}, we attempted to design structures that would be cleaved less efficiently; this would allow better knowledge of cell extract proteins able to interact with such structures. Flap structures not cleaved by FEN1 or sufficiently stable to its action are of special interest for study of BER protein interaction in systems reconstructed from the purified proteins as well as in cell extracts under physiological conditions, that is, in the presence of Mg^{2+}.

MATERIALS AND METHODS

In this study we used dNTP, EDTA, Tris, TEMED, imidazole, SDS, ammonium persulfate, and Coomassie G-250 and R-250 from Sigma (USA); $MgCl_2$, formamide, and NP-40 from Fluka (Switzerland); acrylamide and glycerol from ICN (USA); Hepes and β-mercaptoethanol from Serva (Germany); N,N′-methylenebisacrylamide from BioRad (USA); [γ-^{32}P]ATP (>110 TBq/mmol) and T4 phage polynucleotide kinase (PNK) from Biosan (Russia). Other reagents were of Russian production.

Recombinant proteins—flap endonuclease 1, AP endonuclease 1, poly(ADP-ribose)polymerase 1, and DNA polymerase β—were isolated as described in [9, 11-13], respectively.

Oligodeoxyribonucleotides without modifications were synthesized by research fellows of the group of oligonucleotide synthesis (Institute of Chemical Biology and Fundamental Medicine, Siberian Branch, Russian Academy of Sciences). The sequences of oligonucleotides used in this study are given in the table.

Oligodeoxyribonucleotides with modifications were synthesized using an ASM-700 automatic DNA synthesizer from Biosset (Russia) and standard synthons from Glen Research (USA) for the phosphoramidite protocol [14]. Diethyleneglycol- and 1,10-decanediol-based inserts were incorporated in oligonucleotides using corresponding phosphoramidite synthons synthesized as described in [15]. Oligonucleotides and their derivatives were isolated by reverse-phase HPLC on a column with LiChrosorb RP-18 from Merck (Germany) in an acetonitrile gradient in 50 mM $LiClO_4$.

Radioactive label was incorporated into the 5′-end of oligonucleotides with PNK [16], and the products were purified by electrophoresis in polyacrylamide gel in the presence of 7 M urea under denaturing conditions [16].

Exo-N-[4-(4-azido-2,3,5,6-tetrafluorobenzylidenehydrazinocarbonyl)butyl-carbamoyl]-2′-deoxycytidine-5′-triphosphate, a photoreactive dCTP derivative, was

Structures of oligonucleotides used in this study

Sequences 5′→3′	Types of DNA structures
U1 CTACTAACTAACCGCC	DNA-1 U1 : T
U2 CTACTAACTAACCGC	DNA-2 U1 : D2 : T
U3* CTACTAACTAACCGCC^{Az}	DNA-3 U1 : D3 : T
T GACGATGATGCTGCGGCGT-TAGTTAGTAG	DNA-4 U1 : D4 : T
D2 GCAGCATCATCGTC	DNA-5 U1 : D5 : T
D3 TCTCTCGCAGCATCATCGTC	DNA-6 U3 : T
D4** TCTCTC-(d)-GCAGCATCA-TCGTC	DNA-7 U3 : D2 : T
D5** TCTCTC-(d)-GC-(e)-AGCA-TCATCGTC	DNA-8 U3 : D3 : T DNA-9 U3 : D4 : T DNA-10 U3 : D5 : T

* Oligonucleotide U3 bears a photoreactive group (C^{Az}) at the 3′-end.
** Oligonucleotides D4 and D5 bear non-nucleotide linkers based on decanediol (d) and diethyleneglycol (e).

synthesized according to [17] and kindly donated by Dr. I. V. Safronov (Institute of Chemical Biology and Fundamental Medicine, SB RAS).

Cleavage of flap structures by flap endonuclease 1. The reaction was performed in buffer containing 30 mM Tris-HCl, pH 8.0, 40 mM NaCl, and 8 mM $MgCl_2$. The reaction mixtures (30 μl) contained 50 nM DNA-3, DNA-4, or DNA-5 substrate and 25 nM FEN1. The reaction temperature was 37°C. For analysis, aliquots 5 μl in volume were taken after 2, 5, 7, 10, and 15 min. The reaction products were analyzed by electrophoresis in 20% polyacrylamide gel under denaturing conditions [16] with subsequent autoradiography. Radioactivity of the products was evaluated using Molecular Imager and Quantity One software from BioRad.

Determination of cleavage position of flap-forming oligonucleotide. Sequence of 5′-^{32}P-labeled oligonucleotides was proved by statistical cleavage at the pyrimidine [18] and purine [19] residues. Statistical cleavage of oligonucleotides at the purine residue was performed by treatment with 2% diphenylamine in 66% formic acid for 35 min at 25°C. After completion of the reaction, diphenylamine was extracted with an excess of diethyl ether and oligonucleotides were precipitated with 2% $LiClO_4$ in acetone. The precipitate was washed with acetone and lyophilized. The site of enzymatic cleavage of oligonucleotides was detected by comparison of electrophoretic mobility of the labeled products of degradation with mobility of the 3′-dephosphorylated products of statistical cleavage of corresponding oligomers at positions of the purine residues. The 3′-ends of the resulting fragments were dephosphorylated with PNK. The reaction mixtures (10 μl) contained 100 mM imidazole, pH 6.0, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, BSA (0.1 mg/ml), 0.25 pmol DNA, and 20 ng of PNK. The reaction time was 30 min at 37°C. The reaction products were analyzed by electrophoresis in 20% polyacrylamide gel under denaturing conditions.

Estimation of stability of DNA duplexes and formation of flap substrates–FEN1 complexes. To the mixtures containing 25 nM DNA, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 12.5% glycerol, BSA (0.1 mg/ml), and 1 mM EDTA, FEN1 was added to the final concentration 0.08 or 0.2 μM. The mixtures were incubated on ice bath for 20 min and analyzed by electrophoresis in 8% polyacrylamide gel under non-denaturing conditions. Electrophoresis was performed for 1 h at 150 V and 4°C.

DNA synthesis catalyzed by Pol β. The reaction mixtures (15 μl) contained 10 nM substrate, 5 nM Pol β, four natural dNTPs at concentration 1 μM each, 50 mM Tris-HCl, pH 8.0, 50 mM KCl, and 10 mM $MgCl_2$. The reaction was performed in the absence of FEN1 or in the presence of 50 or 500 nM FEN1 for 10 min at 37°C. The reaction products were analyzed by electrophoresis in 20% polyacrylamide gel under denaturing conditions with subsequent autoradiography. Radioactivity of the products was evaluated using Molecular Imager and Quantity One software from BioRad.

3′-5′-exonuclease activity of apurinic/apyrimidinic endonuclease 1 (APE1). The reaction mixtures (20 μl) contained 5 nM substrate, 3 nM APE1, BSA (0.1 mg/ml), 20 mM Hepes-KOH, pH 8.0, 25 mM KCl, and 0.5 mM $MgCl_2$. The reaction time was 10 min at 37°C. The reaction products were analyzed by electrophoresis in 20% polyacrylamide gel under denaturing conditions.

Design of photoreactive DNA structures. A photoreactive dCTP analog was incorporated into the 3′-end of U2 primer in buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM $MgCl_2$, and 0.25 mM β-mercaptoethanol. The reaction mixture also contained 0.4 μM T template and 0.2 μM 5′-^{32}P-labeled U2 primer. To prepare DNA duplex, primer and template were annealed in a ratio 1 : 2. After formation of DNA duplex, Pol β and the photoreactive dCTP analog were added to the mixture to the final concentrations 1 and 10 μM, respectively. The reaction time was 30 min at 37°C. The DNA sequence provided dCMP incorporation. DNA was precipitated with ten volumes of 2% $LiClO_4$ in acetone, incubated for 40 min at –40°C, and then centrifuged. The pellet was washed with acetone, dried in air at room temperature, and dissolved in TE buffer. To form the substrates, the radioactively labeled photoreactive U3 oligonucleotide was annealed with T template and D2, D3, D4, or D5 oligonucleotides in ratio 1 : 2 : 2 in buffer containing 30 mM Tris-HCl, pH 8.0, and 40 mM NaCl.

Photoaffinity modification of proteins. The reaction mixtures (10 μl) contained 0.03 μM photoreactive DNA substrate, 0.03 μM protein (APE1, FEN1, Pol β, PARP1, or XRCC1), 50 mM Tris-HCl, pH 8.0, and 50 mM KCl. After incubation on ice bath for 5 min, the reaction mixtures were UV-irradiated using a DRK-120 high-pressure mercury lamp and a UFS-6 light filter ($\lambda > 300$ nm, $W = 10^{-4}$ Wt/cm^2, distance 8 cm, irradiation time 5 min). Then the mixtures were analyzed by electrophoresis in 10% polyacrylamide gel according to Laemmli [20] with subsequent autoradiography. Radioactivity of the products was evaluated using Molecular Imager and Quantity One software from BioRad.

RESULTS AND DISCUSSION

Effect of DNA structure on efficiency of cleavage by FEN1. To study the effect of changes in the DNA flap strand on efficiency of the substrate cleavage by FEN1, we used chimeric nucleotide consisting of two sequences bridged by a decanediol linker (these sequences are presented in the table). The linker comparable in length with two nucleotides was located in the 5′-flap strand from the first nucleotide paired with the template. The results of electrophoretic separation of the reaction products of DNA cleavage by FEN1 are presented in Fig. 1a. As shown in the plot (Fig. 1b), the cleavage rate of DNA-4 chimeric structure by flap endonuclease is lower than that of the natural DNA (DNA-3). An additional modification located after the second base paired with the template has been inserted into the flap-forming oligonucleotide. A diethyleneglycol residue was used as a modifying unit (DNA-5). The cleavage rate of flap structures containing two non-nucleotide linkers drastically decreased (Fig. 1a, lanes *11-15*). The level of cleavage of chimeric nucleotide did not exceed 10% of that for the natural oligonucleotide (Fig. 1b).

To determine excision points in the flap-forming oligonucleotides, we compared electrophoretic mobility of the products of FEN1 reaction and that of the products of oligonucleotide cleavage at positions of the purine and pyrimidine residues [18, 19]. On DNA cleavage via this pathway, fragments with the phosphate group at the 3′-end are formed, whereas in the reaction catalyzed by FEN1, oligonucleotides with the hydroxy group at the 3′-end are formed. The presence of the phosphate group affects the electrophoretic mobility of oligonucleotides in polyacrylamide gel, and this can hinder detection of the excision point of the nucleotide chain, accounting for the change in electrophoretic mobility of oligonucleotides with non-nucleotide linkers. That is why after cleavage at dGMP and dAMP residues, the DNA was treated with PNK possessing 3′-phosphatase activity [21]. The results of electrophoretic separation of the reaction products are presented in Fig. 2a. For the natural DNA-3 structure, the cleavage point was after the sixth nucleotide from the 5′-end (Fig. 2b). Chimeric nucleotide was cleaved after the first nucleotide paired with the template.

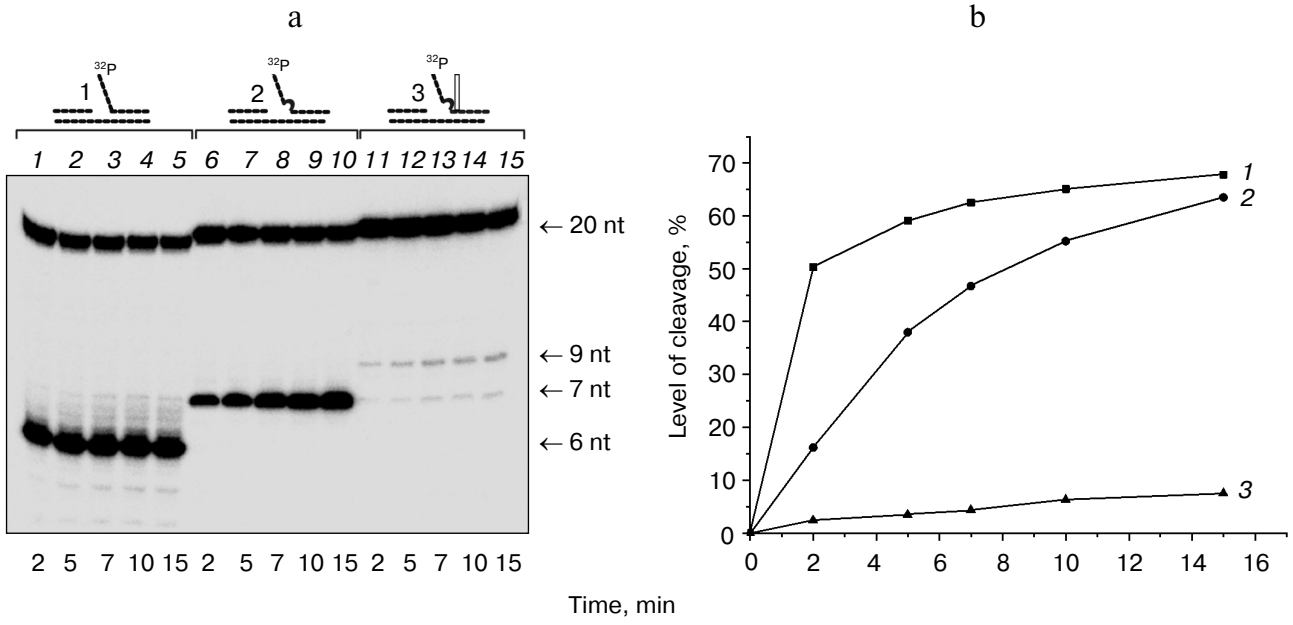

Fig. 1. Effect of DNA structure on efficiency of its cleavage by FEN1. a) Autoradiograph of reaction products after their separation in 20% polyacrylamide gel under denaturing conditions. The reaction mixtures contained 50 nM DNA-3 (lanes *1-5*), DNA-4 (lanes *6-10*), or DNA-5 (lanes *11-15*) substrates and 25 nM FEN1. The reaction was performed at 37°C. Here and in Fig. 2a, arrows designate the length of nucleotides. b) Kinetic curves of cleavage of flap structures by FEN1: *1*) DNA-3; *2*) DNA-4; *3*) DNA-5.

It should be noted that at increased FEN1 concentration, an additional product appears corresponding to the cleavage after the fifth nucleotide from the 5′-end (Fig. 2a, lane *9*).

On replacement of diethyleneglycol residue by the decanediol linker, that is, using oligonucleotide with two decanediol linkers, cleavage efficiency did not change compared with DNA-5 (data not shown); consequently, decreased FEN1 activity was caused not by the nature of the non-nucleotide linker but its presence.

Non-nucleotide linkers can decrease stability of DNA duplexes [22]; as a result, free labeled flap-forming oligonucleotides, which cannot be processed by FEN1, may be present in the reaction mixtures. Decreased activity of FEN1 towards the structures with non-nucleotide linkers can also be caused by less efficient formation of FEN1 complexes with such DNA. Contributions of these factors in decreased efficiency of cleavage of flap structures can be evaluated by electrophoresis of the reaction mixtures under non-denaturing conditions; this allows detection of the enzyme–DNA complexes and determination of the integrity of flap structures. As shown in Fig. 3 (lanes *2-4*), for all DNA substrates the flap-forming oligonucleotide was almost completely within the duplex. Bivalent metal cations are necessary for FEN1 enzymatic activity; however, FEN1 is able to form complexes with flap structures in the absence of cations, but the substrate is not cleaved. Formation of FEN1 complexes with flap structures bearing the non-nucleotide linkers is somewhat less efficient (Fig. 3, lanes *5-7*). However, very low activity of FEN1 towards DNA-5 cannot be caused only by efficiency of complex formation, because the amount of FEN1 complexes with DNA-5 and DNA-4 is almost the same, whereas efficiencies of cleavage of these structures are significantly different. So, although insertion of non-nucleotide linkers into the flap-forming oligonucleotide results in decreased amount of complexes, increased stability of the chimeric structures towards the action of FEN1 is caused not only by worse enzyme-binding characteristics, but also by decreased efficiency of the catalytic stage of DNA cleavage by flap endonuclease.

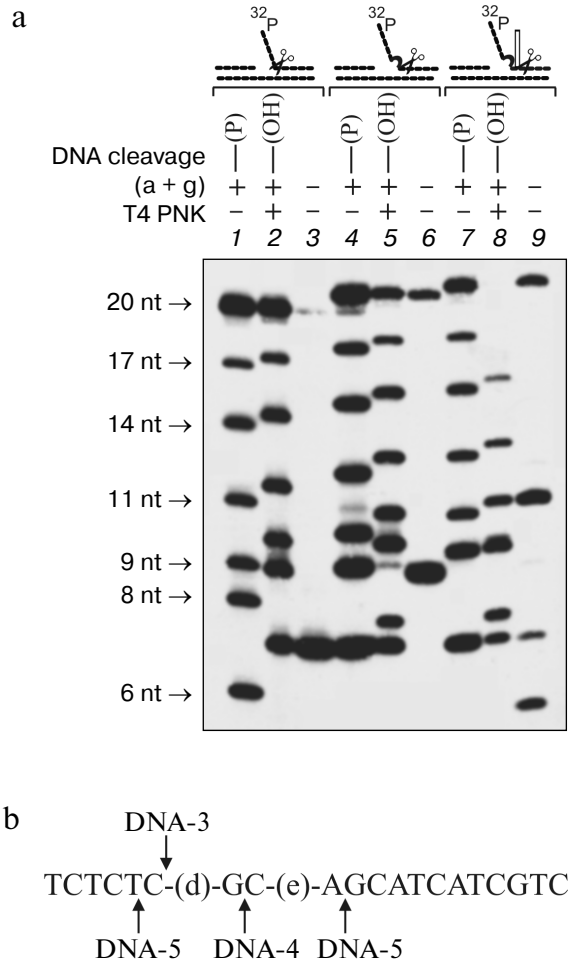

Fig. 2. Determination of cleavage point of the flap-forming oligonucleotide. a) Autoradiograph of reaction products after their separation in 20% polyacrylamide gel under denaturing conditions. The reaction products of DNA cleavage by FEN1 (lanes *3*, *6*, *9*) were compared with the products of oligonucleotide cleavage at positions of the purine residues (lanes *1*, *2*, *4*, *5*, *7*, *8*). To remove the phosphate groups from the 3′-end of oligonucleotides, the DNA fragments were treated with PNK (lanes *2*, *5*, *8*). b) Flap-forming oligonucleotide. Cleavage points are marked by arrows.

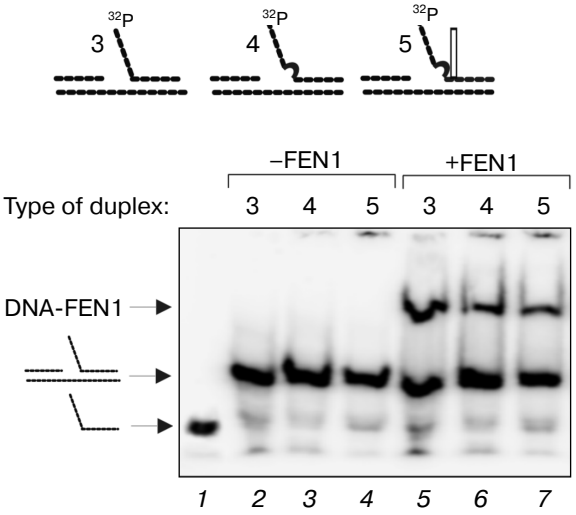

Fig. 3. Evaluation of stability of DNA duplexes and formation of FEN1–flap substrate complexes. Autoradiograph of reaction products after separation in 8% polyacrylamide gel under non-denaturing conditions. The reaction mixtures contained 25 nM DNA in the absence (lanes *2-4*) and in the presence (lanes *5-7*) of 0.08 μM FEN1. Oligonucleotide D5 was used as a control (lane *1*).

Effect of DNA structure on Pol β-catalyzed synthesis and 3′-endonuclease activity of APE1. To evaluate efficiency of recognition and processing of the chimeric flap structures by other BER enzymes compared with the nat-

ural DNA duplexes, we determined the rate of DNA synthesis catalyzed by Pol β and the rate of nucleotide excision at the 3′-end of the initiating primer by the exonuclease activity of APE1. The efficiency of strand-displacement DNA synthesis catalyzed by Pol β was shown to be higher for flap structures containing non-nucleotide linkers (Fig. 4, lanes *4* and *5*). The presence of a linker possibly changes stability of DNA duplexes and results in formation of flap structures with a gap. It is known that gap-bearing DNA duplexes are better Pol β substrates than nick-bearing structures [23]. It should be noted that DNA-3 flap structure (Fig. 4, lane *3*) and DNA duplex with the protruding template strand (lane *1*) are also better Pol β substrates than a nick-bearing duplex (lane *2*). As shown earlier [24], FEN1 stimulates strand-displacement DNA synthesis catalyzed by Pol β on the nick-bearing DNA duplexes with a tetrahydrofuran residue or dRP group at the 5′-end presented into a nick. A "Hit and Run" mechanism has been presented recently [25]. According to this mechanism, in the initial steps of the long-patch BER, after the first nucleotide is incorporated by Pol β, FEN1 cleaves the nick-bearing DNA duplex with the modified ribose phosphate at the 5′-end; as a result, a gap-bearing structure is formed, which is the best substrate for Pol β [23]. Increased degree of synthesis by Pol β in the presence of FEN1 was also observed for flap structures with a flap of one nucleotide in length with a tetrahydrofuran residue at the end of the latter [25]. We studied the effect of FEN1 on Pol β activity towards the nick-bearing DNA duplex, DNA duplex with the protruding template strand, and the flap structures. FEN1 does not effect Pol β-catalyzed DNA synthesis on DNA-1 structure (Fig. 4a, lanes *1*, *6*, *11*). For other structures, efficiency of synthesis increased significantly in the presence of flap endonuclease (Fig. 4b). Stimulation of synthesis for the structures processed by FEN1 and the absence of stimulation for DNA duplex with the protruding template strand, which is not a FEN1 substrate, indicate that increased Pol β activity in the presence of FEN1 is caused not by protein–protein interactions but by the fact that FEN1 generates structures that are better substrates for Pol β.

APE1 is known to possess 3′-exonuclease activity [26]. This activity is ascribed to the proofreading function of APE1 in the BER process. As shown, the exonuclease APE1 activity towards the chimeric structures is less than that towards a normal flap structure (Fig. 5, lanes *3-5*). The maximal excision level was towards the nick-bearing DNA duplex (lane *2*). Insertion of non-nucleotide linkers in the DNA strand seems to change the "geometry" of the DNA duplexes, with resulting changes in their recognition by AP endonuclease.

Thus, DNA duplexes with non-nucleotide linkers can be used in the reconstructed systems containing flap endonuclease, to mimic the intermediates of the long-patch pathway of BER.

Use of chimeric structures for photoaffinity modification of recombinant proteins. We designed photoreactive DNA based on chimeric oligonucleotides, in which photoreactive dCMP was inserted into the 3′-end of initiating primer by activity of Pol β, and a radioactive label was at the 5′-end of the initiating primer. For photoaffinity modification of proteins of the BER system, we used DNA structures of several types: DNA duplex with protruding template strand (DNA-6), nick-bearing DNA duplex (DNA-7), and flap structures (DNA-8, DNA-9, DNA-10) in which the flap-forming oligonucleotide was a normal DNA strand or contained non-nucleotide link-

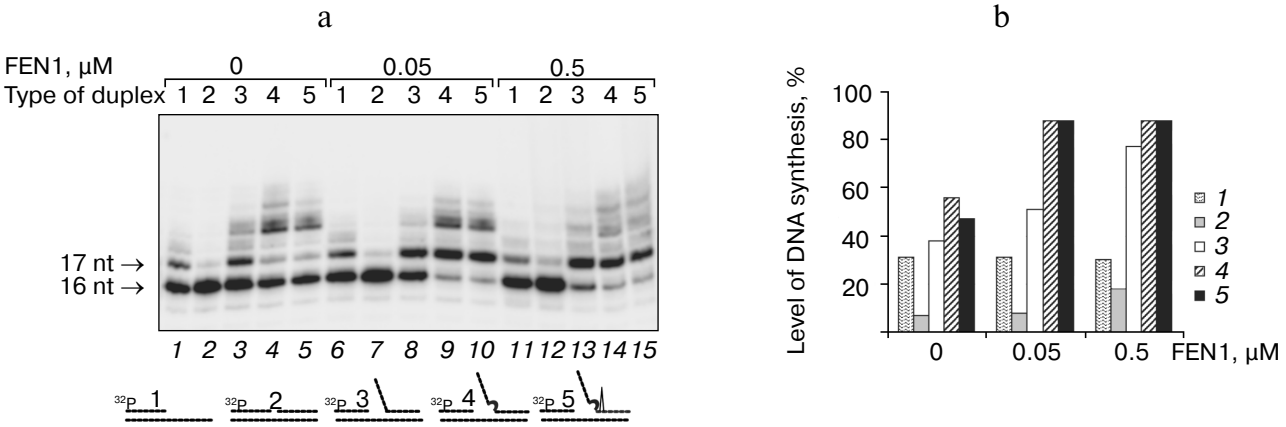

Fig. 4. Pol β-catalyzed DNA synthesis. a) Autoradiograph of reaction products after their separation in 20% polyacrylamide gel under denaturing conditions. The reaction mixtures 15 μl in volume contained 10 nM substrate, 5 nM Pol β, four dNTP (each at 1 μM concentration), 50 mM Tris-HCl, pH 8.0, 50 mM KCl, and 10 mM $MgCl_2$. The reaction was performed in the absence (lanes *1-5*) and in the presence of 50 nM (lanes *6-10*) and 500 nM (lanes *11-15*) FEN1 for 10 min, at 37°C. Radioactivity of the products was determined using Molecular Imager. b) Quantitative estimation of efficiency of Pol β-catalyzed DNA synthesis in the presence of FEN1: *1-5*) from DNA-1 to DNA-5, respectively.

ers. A dCTP derivative, exo-N-[4-(4-azido-2,3,5,6-tetrafluorobenzylidenehydrazinocarbonyl)butyl-carbamoyl]-2′-deoxycytidine-5′-triphosphate, possessing good photochemical characteristics and substrate properties in reaction of DNA synthesis [13, 17] was chosen as the photoreagent. Its structural formula is presented in Fig. 6a. Since flap structures are cleaved by FEN1 in the presence of Mg^{2+} and photoreactive dCMP derivatives can be excised by the 3′-exonuclease APE1 activity [27], experiments on photoaffinity modification were performed under "magnesium-free" conditions. The autoradiograph shows that the level of formation of covalent adducts with Pol β for flap structures is somewhat higher than for DNA-6 and DNA-7 (Fig. 6b, lanes *1-5*). However, efficiencies of the enzyme modification do not significantly differ depending on the structure of flap strand of the oligonucleotide. As mentioned earlier, Pol β synthesizes DNA on the structures with a flap strand more efficiently (Fig. 4).

For APE1, the minimal level of modification is observed for a nick-bearing DNA duplex (Fig. 6b, lanes *6-10*). This structure is the best substrate for 3′-exonuclease reaction. APE1 activity is due not only to the enzyme affinity to DNA, but also by kinetic parameters [28]. As expected, the maximal level of FEN1 modification is provided by flap structures—substrates for this enzyme (Fig. 6b, lanes *11-15*). For chimeric structures, the level of modification is somewhat higher than for DNA-8. For PARP1, the maximal level of modification is observed when DNA duplex with protruding template strand is used (Fig. 6b, lanes *16-20*).

XRCC1 is considered to play a key role in coordination of certain stages of the short-patch pathway of BER via protein–protein interactions [29]. However, data on direct XRCC1–DNA interactions are practically absent. Direct interaction of XRCC1 with gap- or nick-bearing DNA duplexes was shown only for a separate domain of protein [30]. The role of this protein in the long-patch pathway of BER is also practically not studied. It is known only that XRCC1 inhibits strand-displacement DNA synthesis catalyzed by Pol β, when gap-bearing DNA duplex is used as a substrate [31]. The data on photoaffinity modification indicate that this protein efficiently interacts with DNA duplex bearing a protruding template strand and with flap structures (Fig. 6b, lanes *21*, *23-25*). The minimal level of XRCC1 modification is observed when nick-bearing DNA duplex is used (lane *22*). Efficient XRCC1 binding to flap structures suggests its participation in the long-patch pathway of BER; this is a subject of further study using specific functional tests.

So, DNA duplexes with non-nucleotide linkers may be used in the reconstructed systems and in cell extracts as analogs of the long-patch BER intermediates in order to study complexes of proteins participating in this process.

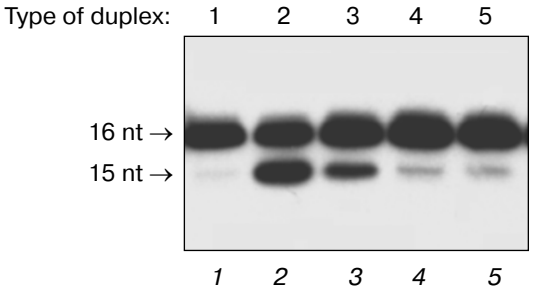

Fig. 5. Effect of the substrate structure on 3′-5′-exonuclease activity of APE1. The reaction mixtures 20 μl in volume contained 5 nM substrate (lanes *1-5* correspond to DNA-1-to-DNA-5, respectively), 3 nM APE1, BSA (0.1 mg/ml), 20 mM Hepes-KOH, pH 8.0, 25 mM KCl, and 0.5 mM $MgCl_2$. Reaction was performed for 10 min at 37°C. The reaction products were analyzed by electrophoresis in 20% polyacrylamide gel under denaturing conditions.

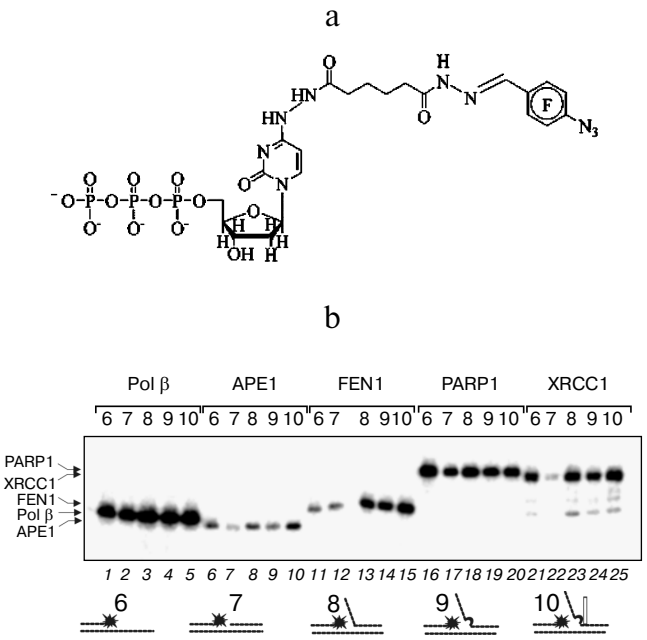

Fig. 6. Photoaffinity modification of the BER proteins. a) Structural formula of dCTP analog used for synthesis of photoreactive DNA. b) Autoradiograph of reaction products after separation in 10% polyacrylamide gel according to Laemmli. The reaction mixtures 10 μl in volume contained 0.03 μM photoreactive DNA substrate, 50 mM Tris-HCl, pH 8.0, 50 mM KCl, and one of the proteins at 0.03 μM concentration: Pol β (lanes *1-5*), APE1 (lanes *6-10*), FEN1 (lanes *11-15*), PARP1 (lanes *16-20*), or XRCC1 (lanes *21-25*). After incubation for 5 min on ice bath, the mixtures were UV-irradiated for 5 min.

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