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# Interaction of Flap Endonuclease-1 and Replication Protein A with Photoreactive Intermediates of DNA Repair

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**Abstract**—A new method for enzymatic synthesis of radioactive DNA flapped structures containing a photoreactive dCMP moiety at a branch point with 4-(4-azido-2,3,5,6-tetrafluorobenzylidene-hydrazinocarbonyl)butylcarbamoyl group attached at *exo*-N-position of cytosine was developed. The formation of complexes of flap endonuclease-1 (FEN-1) with flapped DNA was shown by photoaffinity modification and gel retardation assays. The substrate properties of the flapped structures with different flap lengths were studied in the reaction of endonuclease cleavage catalyzed by FEN-1. It was demonstrated that inhibition of FEN-1 activity by replication protein A (RPA) depends on the length of the single-stranded part of the flapped substrate. A significant inhibition of cleavage was observed when the flap length was sufficient for effective RPA binding, while for structures with short single-stranded part the efficiency of cleavage was independent of the presence of RPA. FEN-1 and RPA were modified by photoaffinity labeling using flap structures with single-stranded parts consisting of 8 and 21 nucleotides. Products of DNA photoattachment to FEN-1 were observed in both cases, while the covalent adducts with RPA were obtained only with the 21-nucleotide-long flap. Photoaffinity modification demonstrated that FEN-1 and RPA compete for the binding of the flapped substrates with long single-stranded parts.

*Key words*: flap endonuclease-1, replication protein A, base excision repair, photoaffinity modification

Genetic stability of an organism depends to a considerable extent on the functioning of the complexes of the enzymes and protein factors that are involved in DNA replication and repair of DNA damage emerging under the influence of exogenous and endogenous factors. The complexes of DNA replication and repair are actually considered as mutually coordinated functional ensembles. The composition of neither complex is fully determined. Several proteins, for example DNA-polymerase δ, replication factor C, PCNA, RPA, and FEN-1 are involved in both processes. The complete identification of the constituents of the complexes of DNA replication and repair in eucaryotes and the evaluation of the protein functions in these processes is one of the important objectives of proteomics.

*Abbreviations*: APE) apurine/apyrimidine endonuclease; FEN-1) flap endonuclease 1; RPA) replication protein A; BER) base excision repair; Pol β) DNA polymerase β; PARP-1) poly(ADP-ribosyl)-polymerase 1; PCNA) proliferating cells nuclear antigen; AP-site) apurine/apyrimidine site.

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There are several different pathways of DNA repair that in particular depend on the type of DNA damage. Base excision repair (BER) is one of the major types of repair of DNA damages [1]. The damaged base is recognized and excised by a specific DNA-glycosylase. Thus, an AP-site is generated. APE cleaves the phosphodiester bond from the 5′-end of the AP-site. Then Pol β can incorporate one nucleotide and eliminate a deoxyribophosphate residue due to its lyase activity followed by the restoration of the integrity of DNA chain by DNA-ligases. In this case DNA will be repaired through the so-called "short patch" pathway. However, if the lyase activity of Pol β is not expressed for some reason, the system switches to the "long patch" pathway of BER. Under these conditions DNA polymerase δ or ε together with PCNA and replication factor C [2, 3] or Pol β carry out strand displacement DNA synthesis [3, 4]. Thus a flapped structure is formed and FEN-1 cleaves the flapping 5′-end close to the point of transition of the single-stranded DNA into DNA duplex. Then the DNA strands are "sewed" together by a ligase. Flapped structures are also formed during DNA replication and recombination [5, 6].

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It is known from the literature that FEN-1 slides through all unpaired DNA region to the branch point, and high molecular weight adducts hinder this sliding. Murante with coworkers [7] have studied the influence of a prokaryotic protein that binds to single-stranded DNA (SSB-protein) on the catalytic activity of bovine flap endonuclease. SSB-protein inhibits FEN-1 activity by binding to the single-stranded part of the flapped structure. RPA is an analog of SSB-protein in eucaryotic cells. There are two different types of RPA binding to the single-stranded DNA [8]. The "8-nucleotide" binding suggests the formation of the unstable RPA complexes with DNA. These complexes can be identified only in the presence of the agents that attach DNA to proteins. When the second type of binding occurs, the RPA molecule changes its conformation and covers over 30 nucleotides, thus forming complexes with significantly higher affinity.

Data concerning the role of RPA in BER are controversial. It was shown that RPA had no influence on the reconstituted system containing BER components [9]. On the other hand, some data indicate that in an *in vitro* system that reconstituted the last BER stage and contained human proteins (FEN-1, DNA-ligase 1, and Pol β) RPA stimulated DNA repair through the long patch pathway [10]. Stimulation of PCNA-dependent repair of AP-sites by RPA was also demonstrated by other investigators using cellular extracts [11]. If RPA in fact stimulates BER, it remains unknown how it occurs and what enzymes are activated by RPA. Work [12] is the only example of the investigation of a direct influence of RPA on the activity of flap endonuclease. This study was carried out with RTH-1, a FEN-1 homolog from yeasts. In the case of a flapped structure with short double-stranded region (14 nucleotides), RPA inhibited RTH1 activity due to melting of the flapped structure. RPA can destabilize DNA duplexes at low salt concentrations [13]. When another substrate was used with circular DNA of M13 phage as a template, in which the length of the double-stranded region was increased to prevent melting of DNA duplex by RPA, and the single-stranded region was shorter than 10 nucleotides, an increase of the catalytic activity of RTH1 was observed when RPA concentration increased. It should be noted that the flapping single-stranded region of this structure was too short to bind RPA with high affinity.

The method of photoaffinity modification is widely applied for the study of specific interactions between biopolymers [14-19]. This method provides valuable information on the structural and functional organization of supra-molecular structures. Incorporation of photoreactive groups in particular DNA structures permits the detection of proteins that selectively interact with these structures [14]. It appears that the most interesting application of this approach is the comprehensive investigation of the functioning of enzymatic assemblies.

Photoreactive DNA with reactive nucleotides in practically in any position of the DNA chain can be obtained by a combination of enzymatic reactions. The ability of DNA-polymerases to use the photoactive analogs of deoxynucleotide triphosphates as the substrates is widely used for the introduction of the photoreactive nucleotides to the 3′-end of DNA [17-19]. In addition, we have shown that oligonucleotides with photoactive group on the 5′-terminal phosphate are obtained in the reaction catalyzed by T4 polynucleotide kinase using substituted γ-amides of ATP as the substrates [20]. Photoreactive nucleotide residues in an internal positions are incorporated step-by-step [21]. First the one-nucleotide-long gap in DNA duplex formed from oligonucleotides is filled with a photoactive residue dNMP using DNA-polymerase. The resulting single-stranded nick is then ligated by T4 DNA-ligase.

It was demonstrated earlier [22] that FEN-1 is modified by structures with a photoreagent on the 5′- or 3′-ends of the oligonucleotides that are oriented towards the single-stranded nick in DNA. The flapped structure with an reactive group located on the 5′-end of the flapping chain or on the 3′-end of the upstream primer was also used as a photoaffinity reagent. The rate of FEN-1 modification by the 3′-terminal reagent was almost the same for both substrates, while FEN-1 labeling by 5′-terminal reagent occurred only in the nick. When the photoactive group was located on the flapping end of the flapped structure, the products of photoattachment to FEN-1 were not observed due to the cleavage of the substrate by the enzyme, and consequently, due to excision of the photoreactive residue from the DNA duplex.

The photoactive substrates with the reactive group located at the point of transition of the flapping single-stranded fragment to DNA duplex are the most prospective structures for the study of interaction of flap endonuclease with the components of the system of the long patch pathway of BER. FEN-1 cleaves DNA close to this branch point [23]. DNA-polymerases that carry out strand displacement DNA synthesis also appear to be in contact with this region [24]. The objectives of this study were the design of photoactive DNA with the reactive group attached to dCMP nucleotide that is located in the branch point of the flapped structure; the photoaffinity modification of FEN-1 and RPA by these structures for the assessment of the peculiarities of interaction of these proteins with each other and with DNA. The photoactive flap structures may be considered as analogs of intermediates of the long patch pathway of BER and can be used in the future for investigation of interactions between the components of the repair complex.

## MATERIALS AND METHODS

The following reagents were used in this study: dNTP, EDTA, Tris, TEMED, imidazole, sodium dodecyl sulfate (SDS), ammonium persulfate, Coomassie G-250

and R-250 (Sigma, USA); $MgCl_2$, formamide, NP-40 (Fluka, Switzerland); glycerol, acrylamide (ICN, USA); β-mercaptoethanol (Serva, Germany); N,N′-methylene-bis-acrylamide (Bio-Rad, USA); DEAE-paper DE-81 (Whatman, England); and [γ-$^{32}$P]ATP (>110 TBq/mmol; Biosan, Russia). The other reagents were from Russian suppliers. The following chromatographic sorbents were used for enzyme purification: phosphocellulose P-11 (Whatman); heparin-Sepharose, Sephacryl S-200 (Pharmacia, Sweden); and $Ni^{2+}$-chelating resin (Invitrogen, USA).

Recombinant RPA and Pol β were isolated as described in [25, 19], respectively. Recombinant T4 polynucleotide kinase containing a cluster of six histidines was expressed in *E. coli* and purified to homogeneity by subsequent chromatographies on $Ni^{2+}$-chelating resin, heparin-Sepharose, and phosphocellulose P-11.

Radioactivity was measured by the Cherenkov method (isotope $^{32}$P) on a Minibeta counter (LKB, Sweden).

All oligonucleotides were synthesized in the group of oligonucleotide synthesis of the Novosibirsk Institute of Bioorganic Chemistry, Siberian Branch of the Russian Academy of Sciences. The 5′-end of oligonucleotide was radioactively labeled using T4 polynucleotide kinase [26]. The oligonucleotides were purified by electrophoresis in a denaturing polyacrylamide gel with 7 M urea [26].

**Photoreactive dCTP derivative** *exo-N*-[4-(4-azido-2,3,5,6-tetrafluorobenzylidene-hydrazinocarbonyl)-butylcarbamoyl]-2′-deoxycytidine-5′-triphosphate was synthesized according to [18].

**Recombinant human FEN-1** was expressed in an *E. coli* system (strain BL21DE3 (pLysE)). Plasmid pET28-FSH (w_t.FE) expressing the FEN-1 gene was kindly provided by Dr. Prassade (National Institute of Environmental Health Sciences, USA). Cell culture transformed with the plasmid was grown at 30°C with continuous aeration until $A_{550}$ = 0.6, then it was chilled and isopropyl-β-D-thiogalactoside was added to the final concentration 0.5 mM. The protein was expressed during 4 h at 25°C. The cells were lysed in buffer (1 ml of buffer for 1 g of cells) containing 50 mM Tris-HCl, pH 8.0, 0.6 M KCl, 2% NP-40, and 7 mM β-mercaptoethanol. DNA was degraded by sonication. The cellular debris was eliminated by centrifugation. The supernatant was applied on a column with $Ni^{2+}$-chelating resin equilibrated with buffer 1 containing 0.3 M KCl, 0.1% NP-40, 3.5 mM β-mercaptoethanol, and 50 mM Tris-HCl, pH 8.0. The column was washed with the same buffer and then with 5 mM imidazole solution in buffer 1. Elution was carried out using buffer 2 containing 200 mM imidazole, 0.3 M KCl, 0.1% NP-40, 2 mM β-mercaptoethanol, 50 mM Tris-HCl, and 10% glycerol. One-milliliter fractions were collected in tubes with 3 ml of buffer 3 (0.1% NP-40, 7 mM β-mercaptoethanol, 50 mM Tris-HCl, 5% glycerol, 2 mM EDTA) to reduce concentrations of imidazole and KCl. The presence of proteins in the fractions was estimated by the Bradford method [27]. The fractions that contained protein were collected together and applied on a column with heparin-Sepharose equilibrated with buffer 3. The column was washed with 2.5 volumes of buffer 3. Elution was carried out in linear KCl gradient (0.1-1 M in buffer 3, total volume 100 ml). Aliquots were taken from protein-containing fractions, and the purity of the preparation was assessed by electrophoresis in polyacrylamide gel according to Laemmli [28]. The selected fractions were pooled, dialyzed against buffer 4 (1 mM β-mercaptoethanol, 50 mM Tris-HCl, 10% glycerol, 0.5 mM EDTA, and 0.1 M KCl) and applied on a column with phosphocellulose P-11 equilibrated with the same buffer. Elution was carried out in a linear KCl gradient (0.1-1 M) in buffer 3. Fractions containing only FEN-1 were collected and dialyzed for 4 h against buffer 4, then 12 h against buffer 5 (1 mM β-mercaptoethanol, 50 mM Tris-HCl, 50% glycerol, 0.5 mM EDTA, and 0.1 M KCl). The purity of the preparation was assessed by electrophoresis in polyacrylamide gel according to Laemmli [28]. Protein concentration was measured according to [27].

**Design of flapped structures with photoreactive group attached to dCMP located in the branch point.** The reaction of billing of the mononucleotide gap by the photoactive dCTP derivative was carried out in the following buffer: 50 mM Tris-HCl, pH 8.8, 50 mM KCl, 10 mM $MgCl_2$, and 0.25 mM β-mercaptoethanol. The reaction mixture also contained 0.75 μM template M, 0.5 μM primer F0 or F1 (for preparation of photoactive oligonucleotides F3 and F4, respectively), and 0.5 μM 5′-[$^{32}$P]-primer F2. For preparation of DNA-duplex primers and the template were mixed in molar ratio 1 : 1.5 in corresponding buffer, transferred into a water bath at 90°C and chilled slowly to room temperature. After the formation of DNA duplex the following components were added to the mixture: Pol β to the concentration of 1 μM, the photoactive dCTP derivative to the concentration 10 μM. The reaction was carried out at 37°C for 30 min. The DNA sequence guaranteed dCTP incorporation. After repair of the gap by the photoactive dCTP analog, 25 units of T4 DNA-ligase for each 10 μl of the mixture and ATP to concentration 1 μM were added to the mixture. The reaction was carried out for 1 h at room temperature. Ligated oligonucleotide was isolated by electrophoresis in denaturing polyacrylamide gel with 7 M urea [26]. Thus were obtained the $^{32}$P-labeled oligonucleotides F3 an F4 with the photoactive dCMP residue in the internal position. For generation of the flapped substrates AzF1-8 and AzF1-21, 25 pmol of oligonucleotides F3 and F4, respectively, were mixed with oligonucleotides T and O (200 pmol each) in buffer containing 30 mM Tris-HCl, pH 8.0, and 40 mM NaCl. The final volume of the reaction mixture was 100 μl. The tube was put into a water

bath at 90°C and then chilled slowly to room temperature. The flapped structures F1-21, F1-8, and F1-4 were obtained analogously using 5′-[$^{32}$P]-oligonucleotides f1-1, f1-2, and f1-3.

**Formation of the complexes of the flapped substrates with FEN-1** was detected by gel retardation assay. FEN-1 was added to the final concentration 0.15, 0.3, 0.9, and 2.1 μM to the mixtures that each contained 0.4 pmol of the substrate AzF1-21 or AzF1-8 in the following buffer: 30 mM Tris-HCl, pH 8.0, and 40 mM NaCl. The final volume of the mixture was 10 μl. The mixtures were incubated on ice for 5 min and then analyzed by electrophoresis in 10% non-denaturing polyacrylamide gel.

**Assessment of RPA influence on FEN-1 activity.** The reaction was carried out in buffer containing 30 mM Tris-HCl, pH 8.0, 46 mM NaCl, and 8 mM $MgCl_2$ (buffer A). The 10 μl reaction mixture also contained 62 fmol of substrates F1-21, F1-8, or F1-4, and 0.5, 1, 2, or 4 pmol of RPA. The mixtures were incubated at room temperature for 5 min. Then 1 pmol of FEN-1 was added to the mixtures. The reaction was carried out at 37°C for 30 min. The reaction products were analyzed by electrophoresis in denaturing 20% polyacrylamide gel [26] followed by radioautography.

**Melting of DNA duplexes by RPA** was verified according to [29]. RPA to final concentration 0.05, 0.1, 0.2, or 0.4 μM was added to the mixtures containing 6.2 nM of substrate (F1-21, F1-8, F1-4) in buffer A. The final mixture volume was 10 μl. The mixtures were first incubated for 5 min at room temperature and then for 30 min at 37°C. Then 1 μl of mixture containing 2.2% SDS, 60% glycerol, and 0.01% bromophenol blue was added. The mixtures were analyzed by electrophoresis in non-denaturing 6% polyacrylamide gel at 4°C.

**Photoaffinity modification of FEN-1 and RPA** was carried out in buffer A in the absence of $Mg^{2+}$. In addition, 10 μl of reaction mixture contained 0.04 μM photoreactive flapped substrate (AzF1-8 or AzF1-21), 0.45 μM FEN-1, and 0.34 μM RPA. The mixture was incubated for 3 min on ice and then irradiated with UV-light for 2 min (wavelength >300 nm, mercury lamp DRK-120, filter UFC-6, $10^{-4}$ W/cm$^2$, distance 8 cm). Afterwards the mixture was analyzed by electrophoresis in 15% polyacrylamide gel according to Laemmli [28] followed by radioautography.

## RESULTS AND DISCUSSION

**Influence of RPA on endonuclease activity of FEN-1.** It known from the literature that the affinity of FEN-1 to flapped structures is higher than to pseudo-Y-structures and double-stranded DNA with gaps and nicks [30]. Structures with flapping single-stranded fragment are formed during DNA repair and replication, the length of the flapping DNA fragment varying over a rather broad range. It was demonstrated earlier [23] that the efficiency of the substrate cleavage by flap endonuclease was independent of the length of the single-stranded fragment. These data were confirmed in the experiment with flapped structures F1-21, F1-8, an F1-4 (the sequences of used oligonucleotides are presented in the table) with flapping regions consisting of 21, 8, and 4 nucleotides, respectively. The length of the duplex-forming region was the same in all substrates. The length of the single-stranded fragment was chosen taking into account the discussed below parameters. Since it was supposed to study the influence of RPA on the efficiency of flap endonuclease interaction with DNA, we took into account the DNA-binding properties of RPA. According to data in the literature, RPA [8] binds to single-stranded DNA in two different ways. The first suggests that RPA binds to 8 nucleotides forming a low-affinity complex. It is the minimal detected length of the "binding box" for RPA. In the second type of binding, the RPA molecule covers over 30 nucleotides with formation of a

Structures of the oligonucleotides used in this study

| | Length | Sequence |
|---|---|---|
| F1 | 21 nt. | 3'-GGACGTCCAGCTGAGATCTCC-5' |
| F0 | 8 nt. | 3'-GGACGTCC-5' |
| F2 | 20 nt. | 3'-ACTAATGCGGTTCGAACGTA-5' |
| M | 24 nt. | 3'-GCTGGACGTCCGTACGTTCGAACC-5' |
| fl-1 | 42 nt. | 3'-ACTAATGCGGTTCGAACGTACGGACGTCCAGCTGAGATCTCC-5' |
| fl-2 | 29 nt. | 3'-ACTAATGCGGTTCGAACGTACGGACGTCC-5' |
| fl-3 | 25 nt. | 3'-ACTAATGCGGTTCGAACGTACGGAC-5' |
| O | 16 nt. | 3'-GAGCTTAAGTGACCGG-5' |
| T | 38 nt. | 3'-GGTCACTTAAGCTCGGTACGTTCGAACCGCATTAGTGG-5' |

much more stable complex. Thirty nucleotides is the maximal length of the single-stranded DNA that interacts with one RPA molecule. According to the data of photoaffinity modification [15, 31, 32], this protein effectively binds to the single-stranded DNA fragments with lengths ranging from 8 to 30 nucleotides by changing its conformation depending on the length of the single-stranded region of DNA-duplex. When the "binding box" is longer than 20 nucleotides, the protein conformation does not change markedly any more. Hence, the lengths of the single-stranded flapping fragments in the flapped structures were chosen in such way that RPA could not bind to the single-stranded fragment (F1-4), or bound to it with low affinity (F1-8), or formed a stable complex (F1-21).

The flap-forming oligonucleotide contained the radioactive label on the 5′-end. We followed the reaction by generation of the radioactive oligonucleotides with lengths that were comparable with that of the single-stranded fragment of the flapped substrate. The rate of transformation of the substrate into the product during 30 min at 37°C for them was approximately 80%.

Interestingly, the position of cleavage of DNA chain by the enzyme was dependent on the length of the unpaired region (Fig. 1). Thus, for the F1-21 substrate with the maximal length of the single-stranded fragment, the cleavage products were represented by the only radioactive oligonucleotide (lanes *11-15*). After the reaction with F1-8 two radioactive bands with different intensities were observed (lanes *6-10*). When F1-4 substrate was used, two radioactive bands with comparable intensities were obtained (lanes *1-5*). We suggest that the increase in the quantity of the product with greater electrophoretic mobility observed when the flap length was reduced deals with the effect of FEN-1 binding to the substrates with short flap that favors partial melting of the DNA duplex. The later event leads to the shift of the position of DNA cleavage.

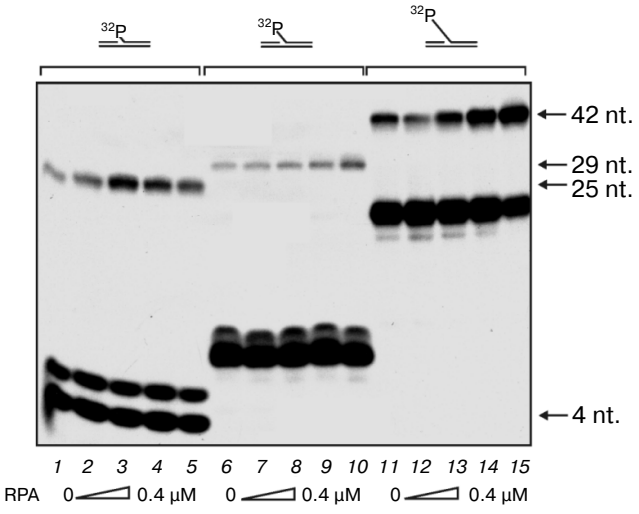


**Fig. 1.** Influence of RPA on the efficiency of cleavage of flapped substrates with different lengths of the flap by FEN-1. The figure represents the radioautograph of the gel after separation of the reaction products in 20% polyacrylamide gel. The reaction mixtures contained 6 nM of the substrates F1-4 (lanes *1-5*), F1-8 (lanes *6-10*), or F1-21 (lanes *11-15*) to which was added RPA to concentration 0 (lanes *1*, *6*, *11*), 0.05 (lanes *2*, *7*, *12*), 0.1 (lanes *3*, *8*, *13*), 0.2 (lanes *4*, *9*, *14*) or 0.4 μM (lanes *5*, *10*, *15*), and the mixtures were incubated for 5 min at room temperature. Then FEN-1 was added to concentration 0.1 μM. The reaction was carried out 30 min at 37°C. The lengths of oligonucleotides are indicated by arrows.

Since the data about the role of RPA in BER are controversial, we studied the influence of RPA on FEN-1 activity using flapped substrates with 4-, 8-, or 21-nucleotide-long flaps (F1-4, F1-8, and F1-21, respectively). Figure 1 (lanes *11-15*) shows that for the 21-nucleotide-long single-stranded fragment of the flapped substrate the increase in RPA concentration leads to reduction in the quantity of the cleavage product. This probably happens due to the competition between RPA and FEN-1 for the single-stranded DNA fragment. The increase in RPA/substrate ratio leads to reduction of the product quantity.

The ability of RPA to form complexes with the indicated structures was assessed by gel retardation assay. Figure 2 demonstrates that RPA can effectively bind only to F1-21 flapped substrate. In this case the major part of the substrate is in complex with RPA (lane *4*). The electrophoretic mobility of this complex in 6% polyacrylamide gel is very low. RPA complexes with F1-8 and F1-4 substrates were not detected (Fig. 2, lanes *5*, *6*). The absence of an influence of RPA on the efficiency of cleavage of the flapped structures with short single-stranded fragments is apparently mediated by the inability of RPA to bind to such substrates.

It was demonstrated earlier that RPA prevents cleavage of the flapped substrate by flap endonuclease from yeast cells. This effect is mediated by the ability of RPA to melt DNA duplexes especially at low salt concentrations [12, 13]. To check if the inhibition of FEN-1 in the presence of F1-21 is in fact mediated by RPA binding to the single-stranded DNA region, but not by duplex melting, the stability of the substrates was assessed under the used conditions. At RPA concentrations 0.05, 0.1, 0.2, and 0.4 μM we did not observe any marked melting of the flapped substrates (data not shown). Consequently, the inhibitory effect of RPA in the case of the substrate with long single-stranded fragment is mediated by the competition between RPA and FEN-1 for binding to the flap.

**Design of flapped structures with a photoreactive group attached to dCMP base located in the branch point.** It appears that the photoactive substrates with reactive group located in the point of transition of the flapping single-stranded fragment into the ordinary DNA duplex are the most promising structures for the investigation of

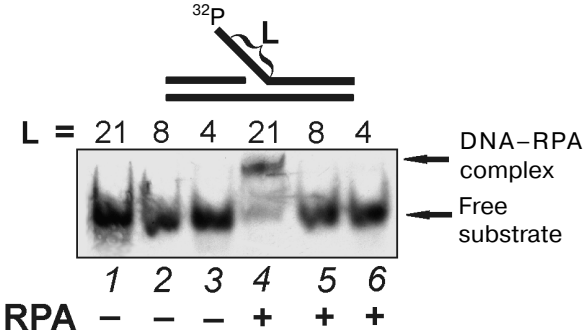


**Fig. 2.** Influence of the length of the single-stranded region of the flapped structure on RPA binding. The figure represents the radioautograph of the gel after separation of the reaction products in non-denaturing 6% polyacrylamide gel. The mixtures contained 6 nM substrates F1-21, F1-8, or F1-4 (lanes *1* and *4*, *2* and *5*, *3* and *6*, respectively), 46 mM NaCl, 8 mM $MgCl_2$, and 0.4 μM RPA (lanes *4-6*). The positions of the flapped substrates and DNA–RPA complex are indicated by arrows.

interactions of flap endonuclease with the components of the long patch pathway of BER (for example, with DNA polymerases). Flap endonuclease cleaves DNA close to this branch point. DNA-polymerases that synthesize DNA with strand displacement also contact with this region. The scheme of the approach applied for design of such structures is presented in Fig. 3. In the first step, a DNA duplex with one-nucleotide gap was formed. In this duplex the oligonucleotide flanking the gap from the 5′-end contained $^{32}$P-labeled phosphate group incorporated using T4 polynucleotide kinase. The mononucleotide gap was filled by photoactive dCTP analog using Pol β under the conditions described in "Materials and Methods". The dCTP analog used for the synthesis of the photoactive flapped substrates was chosen according to its photochemical characteristics and substrate properties in the reaction of DNA synthesis catalyzed by Pol β. The dCTP derivative—*exo-N*-[4-(4-azido-2,3,5,6-tetrafluorobenzylidene-hydrazinocarbonyl)butylcarbamoyl]-2′-deoxycytidine-5′-triphosphate (Fig. 3)—is highly photoactive in the range of 303-313 nm and has good substrate properties in the reaction of DNA synthesis [19]. The use of light with wavelength 300 nm for activation of the photoreagent allows for significant reduction of protein and nucleic acid damage induced by their own photoreactivity in the range of shorter wavelengths. The resulting single-stranded gap was then ligated by T4 DNA-ligase. After the separation of the components by electrophoresis, the ligated oligonucleotide was isolated from the gel by electroelution. The resulting pure $^{32}$P-labeled oligonucleotides with photoactive group were then used for the generation of the flapped substrates AzF1-21 and AzF1-8 with flapping 21- and 8-nucleotide-long single-stranded regions, respectively.

FEN-1 can form complexes with the flapped structures in the absence of bivalent metal ions [30]. Under these conditions the substrate is not cleaved. The presence of such complexes with photoactive structures was confirmed by gel retardation assay (Fig. 4). When the enzyme concentration was increased to 2.1 μM almost all substrate was in the complex with the enzyme (lane *4*). Under these conditions a large quantity of high molecular weight product was formed (lanes *4* and *5*). Its mobility in the gel was significantly lower than that of the com-

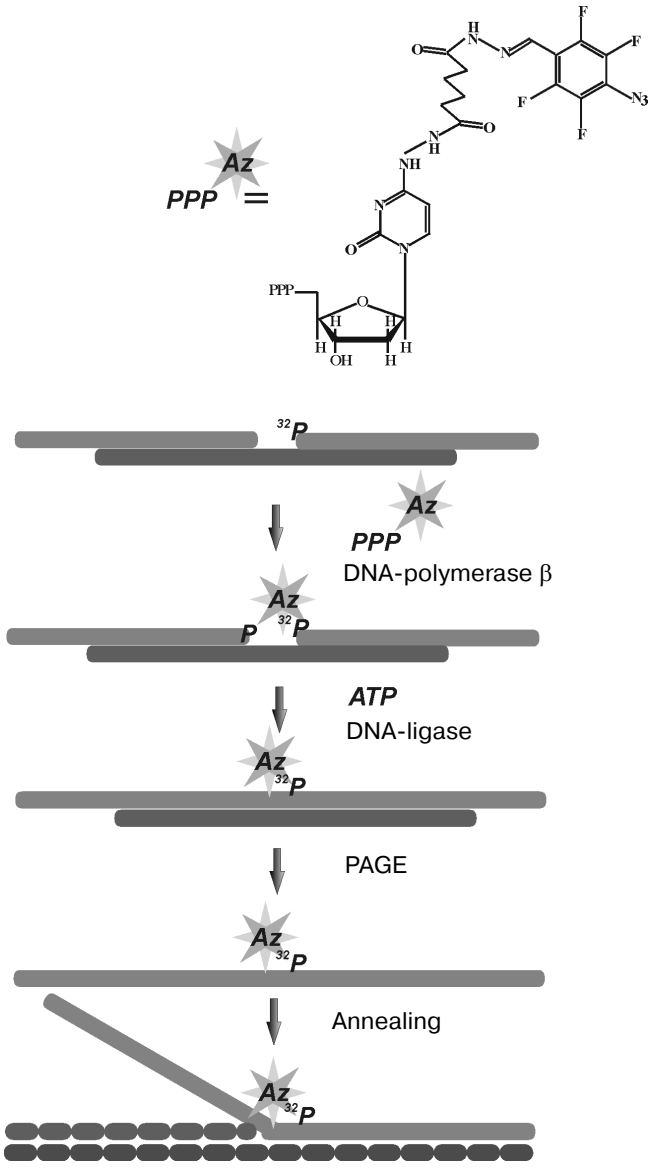


**Fig. 3.** Scheme of generation of the radiolabeled photoactive flapped structures.

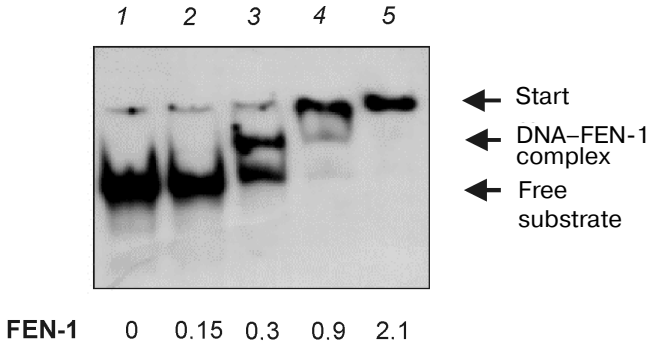


**Fig. 4.** Formation of FEN-1 complexes with the flapped substrate AzF1-21 in the absence of $Mg^{2+}$. The figure represents the radioautograph of the gel after separation of the reaction products in non-denaturing 10% polyacrylamide gel. The reaction mixtures contained 30 mM Tris-HCl, pH 8.0, 40 mM NaCl, 0.04 μM substrate AzF1-21, and the enzyme at concentration indicated on the figure. The positions of DNA substrate and DNA–enzyme complex are indicated by arrows.

plex, in which one molecule of the substrate corresponds to one FEN-1 molecule (lane *3*). This "supershift" was observed earlier at high protein concentrations for other enzymes, for example, for HIV reverse transcriptase [33].

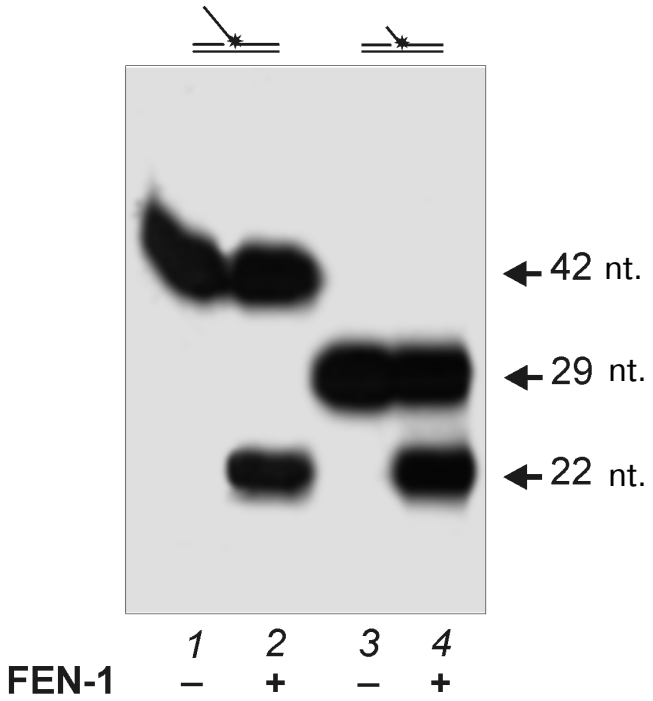


**Fig. 5.** Cleavage of the photoactive flapped substrates by FEN-1. The figure represents the radioautograph of the gel after separation of the reaction products in denaturing 20% polyacrylamide gel. The reaction was carried out for 30 min at 37°C in buffer containing 30 mM Tris-HCl, pH 8.0, 40 mM NaCl, 8 mM $MgCl_2$. The mixture contained 6 nM substrate AzF1-21 (lanes *1*, *2*) or AzF1-8 (lanes *3*, *4*) and 0.1 μM FEN-1. The lengths of oligonucleotides are indicated by arrows.

It is possible that in great excess of the enzyme with respect to the substrate complexes containing more than one enzyme molecule are formed.

Figure 5 presents the results of cleavage of the photoreactive substrates AzF1-21 and AzF1-8. In these substrates the radioactive phosphate is located between the first and second nucleotides paired to the template. The cleavage of both substrates leads to the formation of radiolabeled products of the same length (lanes *2* and *4*). This observation provides evidence that FEN-1 cleaves the substrate at the 5′-end from the paired with the template nucleotide. Additional data about the position of cleavage of the photoactive flapped structures were obtained using the analogous flapped structures where oligonucleotide O (see the table) was elongated from the 3′-end for one dCMP residue. The generated flapped structure had a pseudo-gap in the point formed by the 3′-end of the O primer and the flap-forming oligonucleotide. In other flapped structures used in this study, a pseudo-gap of one nucleotide was formed in the same position. The treatment of both substrate types by flap endonuclease led to the generation of radioactive products of the same length. Subsequent incubation of these reaction mixtures in the presence of T4 DNA-ligase resulted in the formation of the ligation product when the flapped structure with a pseudo-nick was used (data not shown). Therefore, taking into account all these data, one can undoubtedly affirm that the photoactive flapped structures are cleaved by flap endonuclease directly in the point of transition of the single-stranded region to the double-stranded part. According to the data in the literature, the position of cleavage of the flapped structures by FEN-1 may differ. Thus, for example, it was shown in several studies that the cleavage of the flapping oligonucleotide with $^{32}P$-label on the 5′-end led to the generation of two radioactive products; the cleavage of DNA chain occurred mainly one nucleotide upstream of the first paired with the template nucleotide, or directly downstream to it [23]. At the same time, in some cases only one product was generated. Its length corresponded to the length of the flapping fragment of the flapped structure [34]. When the experiments are carried out in systems that are reconstituted from purified proteins [3], the activity of flap endonuclease results in the formation of oligonucleotides that are then ligated by DNA-ligase. The comparison of cleavage of the photoactive flapped substrates AzF1-8 and AzF1-21 with analogous, but not photoactive, flapped structures F1-8 and F1-21 revealed some differences. F1-8 was cleaved in two positions (Fig. 1, lanes *6-10*), while AzF1-8 was cleaved only at one point (Fig. 5, lane *4*). The flapped structures AzF1-21 and F1-21 were both cleaved at one point (Fig. 5, lane *2* and Fig. 1, lanes *11-15*). Flap endonuclease effectively binds and cleaves the flapped structures with the photoreactive group in the branch point. This suggests that these structures are analogous to the intermediates of the long patch BER pathway. The

analogous structures are intermediates in processing of Okazaki fragments [35]. Hence, the resulting photoreactive structures may be used for the study of interactions between different proteins involved in DNA replication and repair.

**Photoaffinity modification of FEN-1 and RPA.** Photoactive flapped structures were used for photoaffinity modification of flap endonuclease. Figure 6 (lanes *2*, *5*) shows that photomodification of FEN-1 in the absence of $Mg^{2+}$ resulted in a band that corresponded to a polypeptide with apparent molecular weight 58 kD. It should be noted that in the presence of $Mg^{2+}$ the total level of FEN-1 modification decreases and the product of its modification with apparent molecular weight 50 kD appears. Possibly it is the product of photoattachment of the oligonucleotide generated after the cleavage of the flapping single-stranded fragment of the oligonucleotide. It was shown earlier that FEN-1 is modified by the structure with a single-stranded nick and the photoreactive group on the 5′-end of the oligonucleotide exposed towards the nick [22]. It is known [30] that FEN-1 is able to bind to such structures and excise one or two nucleotides due to its exonuclease activity.

Photoreactive flapped structures used in this study have certain advantages in comparison to the structures that were used earlier [22], since when the complex with FEN-1 is formed, the reactive group is located close to the active center of the enzyme.

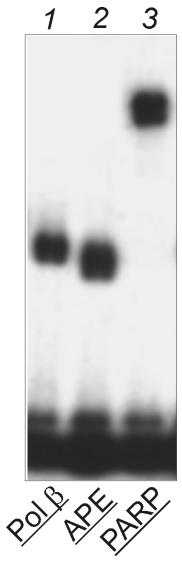


**Fig. 7.** Photoaffinity modification of proteins using photoactive flapped substrate with short single-stranded region. The figure represents the radioautograph of the gel after separation of the reaction products by electrophoresis according to Laemmli in 15% polyacrylamide. Reaction mixtures contained 0.04 μM flapped substrate AzF1-8 and one of the following proteins: 1.50 μM Pol β, 0.41 μM APE, or 0.40 μM PARP-1. The reaction was carried out in buffer containing 30 mM Tris-HCl, pH 8.0, and 40 mM NaCl (lanes *1*, *2*) or 70 mM NaCl (lane *3*).

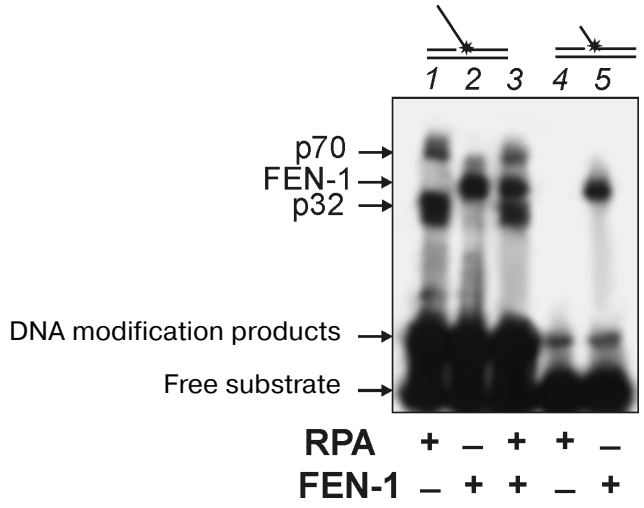


**Fig. 6.** Influence of the structure of the flapped substrate on the efficiency of FEN-1 and RPA modification. The figure represents the radioautograph of the gel after separation of the reaction products by electrophoresis according to Laemmli in 15% polyacrylamide. Reaction mixtures contained 0.04 μM flapped substrate AzF1-21 (lanes *1-3*) or AzF1-8 (lanes *4*, *5*), 0.45 μM FEN-1, and 0.34 μM RPA. The reaction was carried out in buffer containing 30 mM Tris-HCl, pH 8.0, and 40 mM NaCl. The positions of bands corresponding to the products of FEN-1 modification, RPA subunits 70 kD (p70) and 32 kD (p32) are indicated by arrows.

The irradiation of RPA in the presence of the photoreactive flapped substrate with 21-nucleotide-long flapping region results in the appearance of the products of intensive labeling of p70 and p32 protein subunits (Fig. 6, lane *1*), while in the case of the substrate with 8-nucleotide flap the products of RPA modification were not observed (Fig. 6, lane *4*). These data are in agreement with the results of the gel retardation assay (Fig. 2) indicating that RPA forms the complex only with the flapped structure with 21-nucleotide flap fragment, while FEN-1 is effectively modified by both structures (Fig. 6, lanes *2* and *5*). When RPA and FEN-1 were simultaneously modified by AzF1-21 substrate the intensities of the bands corresponding to these proteins decreased in comparison to the controls (separate modification of each protein). This observation indicates that RPA and FEN-1 compete for the substrate with long single-stranded region. Addition of RPA did not change the level of FEN-1 modification by AzF1-8 structure (data not shown).

It was demonstrated using human and hamster cell extracts that *in vitro* the length of DNA fragment that is resynthesized during base repair through the long patch pathway varied from 2 to 10 nucleotides (mean length 6.6 nucleotides) [36]. It is known that the length of the flapping fragment has no influence on the efficiency of cleav-

age of the flapped structures by FEN-1 [23]. Consequently, FEN-1 cannot be a factor that limits the length of the resynthesizing fragment during BER. Inhibition of cleavage of the flapped structures with long flap induced by RPA is possibly one of the factors that determine the length of the resynthesizing DNA fragment. RPA can block repair by binding to long flaps. At the same time, the flapped structures with single-stranded flapping regions shorter than 10 nucleotides bind RPA with lower efficiency, and consequently, the inhibition of cleavage of the flapped structures by flap endonuclease must not occur.

The suggestion that RPA is a factor that regulates the length of the synthesizing fragment in the synthesis with strand-displacement by polymerase δ during processing of Okazaki fragments is discussed in [35].

The photoreactive flapped substrates were also used for photoaffinity modification of several proteins participating in BER. The roles of Pol β and APE in BER were discussed above. Lavrik and coworkers [14] have shown that PARP-1 is involved in BER through the long patch pathway. The data on modification of these proteins are presented in Fig. 7, demonstrating that Pol β, APE, and PARP-1 are effectively modified by the designed flapped structures.

Thus, we proposed an approach for design of flapped structures with a photoreactive nucleotide in the branch point. We also demonstrated the promising role of these structures as analogs of intermediates of the long patch BER pathway for the study of protein complexes that are involved in this process.

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