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Interaction of Nucleotide Excision Repair Factors RPA and XPA with DNA Containing Bulky Photoreactive Groups Imitating Damages

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Abstract—Interaction of nucleotide excision repair factors—replication protein A (RPA) and *Xeroderma pigmentosum* complementing group A protein (XPA)—with DNA structures containing nucleotides with bulky photoreactive groups imitating damaged nucleotides was investigated. Efficiency of photoaffinity modification of two proteins by photoreactive DNAs varied depending on DNA structure and type of photoreactive group. The secondary structure of DNA and, first of all, the presence of extended single-stranded parts plays a key role in recognition by RPA. However, it was shown that RPA efficiently interacts with DNA duplex containing a bulky substituent at the 5′-end of a nick. XPA was shown to prefer the nicked DNA; however, this protein was cross-linked with approximately equal efficiency by single-stranded and double-stranded DNA containing a bulky substituent inside the strand. XPA seems to be sensitive not only to the structure of DNA double helix, but also to a bulky group incorporated into DNA. The mechanism of damage recognition in the process of nucleotide excision repair is discussed.

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Key words: replication protein A, nucleotide excision repair factors, photoaffinity modification, photoreactive oligonucleotides, complexing

In the living cell, DNA is constantly damaged by the action of endogenous reactive metabolites as well as exogenous factors, such as chemical substances and irradiation [1]. A wide variety of repair mechanisms and their control during the cell cycle provide integrity of DNA, preventing subsequent genetic changes, especially mutations and necrosis resulting in tumor genesis and aging, respectively [2-5].

Nucleotide excision repair (NER) is one of the most important mechanisms of DNA repair. This process removes various damages distorting the double helix of DNA, for example, pyrimidine dimers formed by UV irradiation or bulky chemical adducts formed by the action of environmental factors or chemotherapy. NER is a multistage process and requires coordinated action of at least 25-30 polypeptides [4]. Removal of a damaged nucleotide is a complex process including sequential recognition of DNA damage by various NER factors; helix opening around a lesion, dual incision of the damaged strand, and subsequent release of a damaged oligonucleotide [5, 6]. The following protein factors participate in recognition of damage and its processing: XPC–hHR23B, TFIIH, *Xeroderma pigmentosum* complementing group A factor (XPA), DDB1/2, XPG, and ERCC1–XPF. Replication protein A (RPA) plays a key role in recognition of DNA damages and their processing. However, data on the specific role of this protein at the

Abbreviations: FAB-dUMP) 5-{[4-(4-azido-2,3,5,6-tetrafluorobenzoylamino)butyl]-aminocarbonyl-carbamoyl-propyloxymethyl}-2′-deoxyuridine-5′-monophosphate; FAP-dUMP) 5-{N-[N-(4-azido-2,5-difluoro-3-chloropyridin-6-yl)-3-aminopropionyl]-trans-3-aminopropenyl-1}-2′-deoxyuridine-5′-monophosphate; FAP-dCMP) exo-N-{2-[N-(4-azido-2,5-difluoro-3-chloropyridin-6-yl)-3-aminopropionyl]-aminoethyl}-2′-deoxycitidine-5′-monophosphate; NER) nucleotide excision repair; RPA) replication protein A; XPA) *Xeroderma pigmentosum* complementing group A factor.

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initial stages of NER process are contradictory. It is still unclear what NER factor is first to recognize a damaged site. Using a new method of UV-irradiation of cells together with labeling by fluorescent antibodies, it was recently shown that XPC–hHR23B complex recognizing damages seems to be especially important for inclusion of subsequent NER factors into the process of damage removal [7]. XPC–hHR23B is necessary for TFIIH placing on a photodamaged site, whereas XPA is not necessary for TFIIH binding to the damaged nucleotide.

Other researchers consider that RPA provides the primary recognition of damages [8, 9]. For certain types of damages, other proteins can also participate in recognition of a damaged nucleotide. Having high affinity to certain types of damage, XPA as a factor seems to verify damages at subsequent NER stages, although the role of this protein as the initial verifier is still not completely developed [10, 11]. RPA is supposed to participate in the NER process simultaneously with XPA. However, it is known that RPA by itself is sensitive to several types of DNA damage. RPA binds with high affinity to DNA bearing damages, which destabilize its structure [12, 13].

Earlier we studied the interaction between RPA and various photoreactive DNA structures modeling intermediates of DNA replication and repair. We found that the affinity of RPA to DNA bearing a bulky photoreactive group at the 5′-terminal ribose residue of the nick is significantly higher than its affinity to DNA with a nonmodified nick or to a complete duplex bearing a photoreactive group inside the strand [14]. We suggested that such structure can imitate a damaged DNA recognized by NER proteins. Recently we showed that DNAs bearing bulky photoreactive nucleotides incorporated into the 3′-end of the nick were subject to processing in the UV repair system of prokaryotes analogous to the nucleotide repair of eukaryotes [15]. The NER system is known to possess wide substrate specificity, removing various types of damages distorting the duplex structure of DNA. It is possible that the presence of a nick in the neighborhood of a bulky group additionally contributes to destabilization of a DNA duplex; this facilitates recognition of the damaged nucleotide by proteins participating in this process. In this work, we compared the interaction of RPA and XPA with nicked DNA structures in which the photoreactive groups were attached to the 3′- or 5′-terminal nucleotides of the nick. The DNA duplexes containing modifications imitating DNA damages are promising for study of the mechanism of nucleotide excision repair, in particular, the key stage of this process, i.e., the initial recognition of damage. DNA modifications modeling damages and at the same time possessing photoreactive activity are of special interest. Use of such DNA derivatives for photoaffinity modification of proteins may allow identification of unstable intermediates of nucleotide repair and evaluation of the role of certain subunits of NER proteins at various stages of this complex process.

MATERIALS AND METHODS

The following reagents were used in this study: [γ-^{32}P]ATP produced in the Laboratory of Radiochemistry of the Institute of Chemical Biology and Fundamental Medicine (ICBFM) of the Siberian Branch of the Russian Academy of Sciences; Rainbow molecular mass markers from Amersham (USA); phage T4 polynucleotide kinase from Sibenzyme (Russia); reagents for electrophoresis and the main components of buffers were either from Sigma (USA) or produced in Russia and were of extra pure grade.

Oligonucleotides used in this study were synthesized at ICBFM using an ASM-800 nucleotide synthesizer from Biosset (Russia) and purified using an RP cartridge from Cruachem (Scotland) on an OPS-201 system for oligonucleotide purification from Biosset. Oligonucleotides bearing a base-attached photoreactive perfluoroarylazide (FAB) group at the 5′-end or inside the strand were synthesized by the solid-phase method. dUTP and dCTP analogs bearing a base-attached photoreactive difluorochlorazidopyridyl (FAP) group, FAP-dUTP and FAP-dCTP, were synthesized as described in [16] and [17], respectively, and kindly provided by S. V. Dezhurov (ICBFM).

Recombinant RPA was isolated from an *Escherichia coli* strain according to the protocol described in [18]. Recombinant XPA was isolated from the *E. coli* strain and kindly provided by A. Eker (Erasmus Medical Center, Rotterdam, The Netherlands). Recombinant DNA polymerase β was isolated from *E. coli* according to the protocol described in [19] and kindly provided by S. N. Khodyreva (ICBFM).

Incorporation of ^{32}P into the 5′-end of oligonucleotide. ^{32}P was incorporated into the 5′-end of oligonucleotides using T4 polynucleotide kinase as described in [20]. The reaction mixture (20 μl) contained 1 μM oligonucleotide, [γ-^{32}P]ATP (100 μCi), and T4 polynucleotide kinase (5 U). The reaction was performed for 30 min at 37°C and left overnight at 4°C. Then the mixture was separated by electrophoresis in 20% polyacrylamide gel under denaturing conditions as described in [21]. Gel portions containing ^{32}P-labeled oligonucleotides were localized by autoradiography. Then oligonucleotides were isolated by electroelution onto DE-81 paper using 25 mM Tris-borate, pH 8.3, as the electrode buffer. The product was eluted from the DE-81 paper with three aliquots (20 μl) of hot 3 M $LiClO_4$. Acetone (1.2 ml) cooled to 4°C was added to the eluate and the mixture was incubated for 1 h at –40°C. The precipitate was centrifuged, washed with two aliquots (1 ml) of acetone cooled to 4°C, dried, and then dissolved in water to the required concentration.

Annealing of DNA structures. For annealing of DNA structures, a mixture of complementary oligonucleotides was heated to 95°C with subsequent slow cooling to room

temperature. To obtain nicked DNA, we used a two-fold excess of one of the primers (not bearing a photoreactive group), in other cases oligonucleotides were taken in equimolar concentrations. The duplexes thus obtained were analyzed by electrophoresis in 10% polyacrylamide gel under non-denaturing conditions (acrylamide/bis-acrylamide = 30 : 1, 25 mM Tris-borate buffer, pH 8.3). In all experiments, the duplex content in the mixture was not less than 95%.

Incorporation of a photoreactive group into the 3′-end. A photoreactive group was incorporated with DNA polymerase β into the 3′-end of 5′-^{32}P-labeled primer in the corresponding DNA duplex. The reaction mixture (10 μl) contained 50 mM Tris-HCl, pH 8.0 (25°C), 50 mM NaCl, 5 mM $MgCl_2$, 5 μM DNA duplex, 5 μM DNA polymerase β, and 50 μM FAP-dNTP. The mixtures were incubated for 60 min at 37°C. The reaction was terminated by heating for 5 min at 95°C. The products were analyzed by electrophoresis in 20% polyacrylamide gel under the denaturing conditions. DNA was salted out by addition of 100 μl of 3 M $LiClO_4$ and 1.2 ml of acetone cooled to 4°C, and the mixture was incubated for 1 h at –40°C. The precipitate was centrifuged, washed with 300 μl of acetone cooled to 4°C, dried, and dissolved in water.

RPA and XPA were photoaffinity labeled in reaction mixture (10 μl) containing the standard components of buffer (50 mM Tris-HCl, pH 8.0 (25°C), 50 mM NaCl, 5 mM $MgCl_2$), 1 μg/μl BSA, 0.1 μM 5′-^{32}P-labeled photoreactive DNA substrate, and the indicated amounts of RPA or XPA. The mixtures were incubated for 20 min at 37°C and then placed on ice. The samples were UV irradiated using a DRK-120 high-pressure mercury lamp (a VIO-1 lighter from LOMO (Russia), distance 110 mm) within the wave range 313-365 nm through a UFS-6 light filter with light intensity $I = 8 \cdot 10^{14}$; irradiation time was 15 min for FAP derivatives and 30 min for the FAB group. The reaction mixtures were separated by electrophoresis in 10% polyacrylamide gel in the presence of SDS (acrylamide/bis-acrylamide = 30 : 1), gels were dried, and the products of modification were analyzed using the Molecular Imager (BioRad, USA) and Quantity One software.

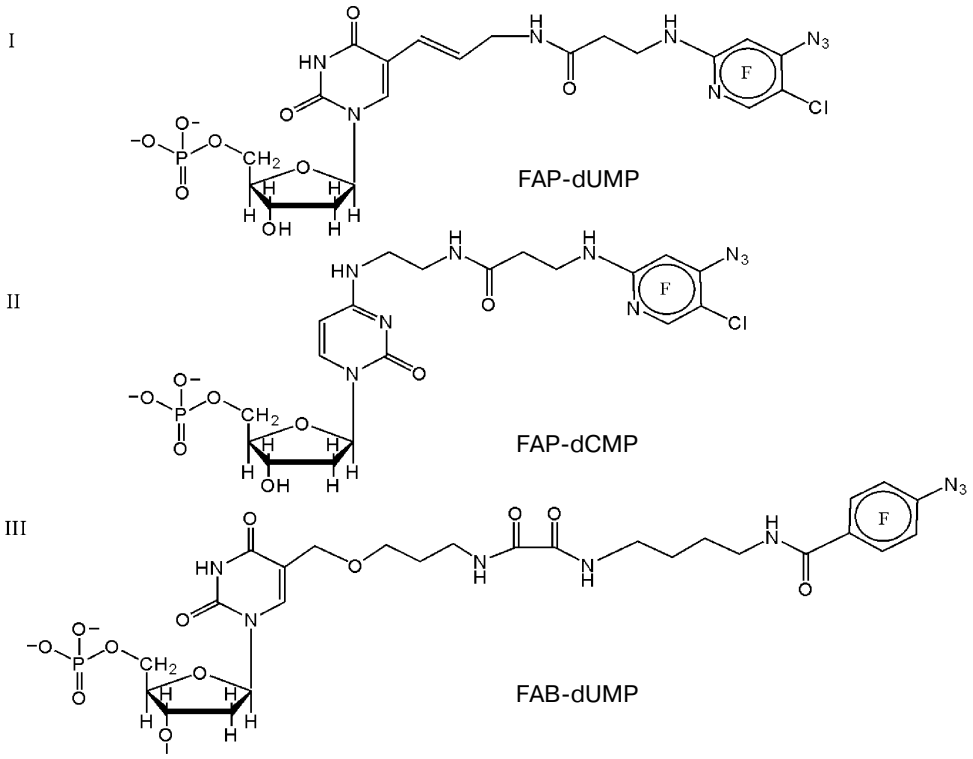
Complex formation of RPA and XPA with DNA was analyzed by gel retardation. The reaction mixture (10 μl) contained 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM NaCl, 5% glycerol, 0.5 μg/μl BSA, 0.01 μM 5′-^{32}P-labeled DNA, and various RPA and XPA concentrations. The mixtures were incubated for 20 min in 37°C and separated by electrophoresis under non-denaturing conditions at room temperature in 4% polyacrylamide gel (acrylamide/bis-acrylamide = 30 : 1; 50 mM Tris-OAc, pH 8.3, 1 mM $MgCl_2$, 0.1% TEMED, 0.05% $(NH_4)_2S_2O_8$). Before loading on the gel, the samples were added with buffer containing 25% glycerol and 0.05% Bromophenol Blue (1/5 volume). Electrophoresis was performed on vertical plates 12 × 10 × 0.1 cm, and 50 mM Tris-acetate, pH 8.3, containing 1 mM $MgCl_2$ was used as the electrode buffer. Samples were loaded onto the gel at 100 V, and electrophoresed at 10 V/cm to 1/2-path migration of Bromophenol Blue. The DNA–protein complexes and free DNA were visualized using BioRad Molecular Imager.

RESULTS AND DISCUSSION

Replication protein A is one of the eukaryotic proteins that is absolutely required for the main processes of DNA metabolism, such as replication, repair, and homologous recombination [22]. Interaction with single-stranded parts of DNA arising in these processes and their stabilization are the main RPA functions [22, 23]. Interaction between RPA and single-stranded DNA is nonspecific and is characterized by high affinity (K_a = 10^8-10^{10} M^{-1}) depending on the length of DNA participating in complex formation [24-27]. The affinity of RPA to double-stranded DNA is usually 2-3 orders of magnitude lower [28, 29]. It is known that RPA is able to interact specifically and efficiently with double helix DNA treated by UV irradiation or such damaging agents as acetylaminofluorene or cis-diaminodichloroplatinum [30-32]. RPA is known to destabilize double-helical DNA under certain conditions; this results in formation of single-stranded DNA parts bound by RPA [28, 33].

As we showed earlier by photoaffinity labeling, RPA is able to interact efficiently with DNA duplex containing a bulky photoreactive perfluoroarylazide group bound through amino linker to the 2′-position of ribose at the 5′-end of the nick [14]. The affinity of RPA to this substrate estimated by gel retardation was 30 times higher than its affinity to complete (without break and modification) DNA duplex of the same length. In this study, we present results of photoaffinity labeling of RPA and XPA by structures bearing a photoreactive group at the 3′- or 5′-end base in the nick, partial DNA duplex with 5′- or 3′-protruding end of template, and also inside the chain of completely or partly complementary duplex. Difluorochloroarylazidopyridyl group bound through the amino linker to the 5′-position of uracil (FAP-dUMP, structure I, Fig. 1a) or to the exocyclic group of cytosine (FAP-dCTP, structure II, Fig. 1a) and also tetrafluoroazidobenzoyl derivative of uracil (FAB-dUMP, structure III, Fig. 1a) were used as the photoreactive group. DNA structures modified at the 3′-end were obtained by enzymatic synthesis using DNA polymerase β and corresponding photoreactive dUTP and dCTP derivatives as substrates in the primer elongation reaction [34]. Oligonucleotides bearing modification at the 5′-end or inside the strand were chemically synthesized. DNA structures used in this study are presented in Fig. 1b. To clarify whether the position of a bulky substituent in the 5′-end nucleotide

a



b

I 5′-GGCGATTAAGTTGGG**U**^{FAP}
3′-CCGCTAATTCAACCCATTGCAGTCCCAGAAGG

II 5′-GGCGATTAAGTTGGG**U**^{FAP}
5′-pAACGTCAGGGTCTTCC
3′-CCGCTAATTCAACCCATTGCAGTCCCAGAAGG, where **U^{FAP}= FAP-dUMP**

III 5′-GGCGATTAAGTTGGG**C**^{FAP}
3′-CCGCTAATTCAACCCGTTGCAGTCCCAGAAGG

IV 5′-GGCGATTAAGTTGGG**C**^{FAP}
5′-pAACGTCAGGGTCTTCC
3′-CCGCTAATTCAACCCGTTGCAGTCCCAGAAGG, where **C^{FAP}= FAP-dCMP**

V ^{FAB}
5′-**U**ACGTCAGGGTCTTCC
3′-CCGCTAATTCAACCCACACAGTGATGCAGTCCCAGAAGG

VI 5′-GGCGATTAAGTTGGGTGTGTCAC ^{FAB}
5′-**U**ACGTCAGGGTCTTCC
3′-CCGCTAATTCAACCCACACAGTGATGCAGTCCCAGAAGG

VII ^{FAB}
5′-GGCGATTAAGTTGGGTGTG**U**CACTACGTCAGGGTCTTCC

VIII ^{FAB}
5′-GGCGATTAAGTTGGGTGTG**U**CACTACGTCAGGGTCTTCC
3′-CCGCTAATTCAACCCACACAGTGATGCAGTCCCAGAAGG

IX ^{FAB}
5′-GGCGATTAAGTTGGGTGTG**U**CACTACGTCAGGGTCTTCC
3′-CCGCTAATTCAACCCA ATGCAGTCCCAGAAGG
GTGTCAC , where **U^{FAB}= FAB-dUMP**

Fig. 1. Structures of photoreactive nucleotide analogs (a) and DNA sequences (b).

effects interaction between RPA and the nicked DNA, in this study we used a dUMP derivative bearing the base-substituted dUMP residue (in our previous study [14] we used a derivative substituted at the 5′-end sugar residue). DNA duplex with 3′-protruding single-stranded end of template was used for comparison.

Experiments on photoaffinity labeling were performed for RPA and XPA. DNA-binding characteristics of XPA are to some extent similar to those of RPA. It is known that both XPA and RPA are able to bind single-stranded DNA; however, XPA has rather high affinity ($K_a \approx 10^7$ M^{-1}) to certain DNA duplexes such as DNA with platinum adducts, some non-complementary bases ("bubbles"), or small loops [12].

The results of photoaffinity labeling of RPA and XPA are presented in Fig. 2. As shown, RPA is more efficiently labeled by the photoreactive nicked DNA duplex than a structure with the protruding end of template; this agrees completely with the earlier data on RPA modification by DNA duplexes with a photoreactive group bound to ribose at the 5′-end nucleotide [14]. Analogously to RPA, XPA also prefers a structure with single-stranded break carrying a bulky substituent at the 5′-end.

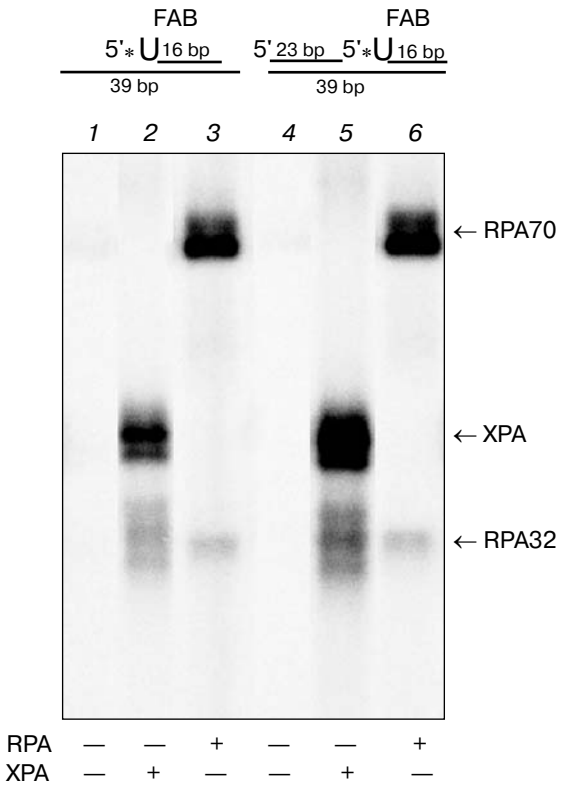


Fig. 2. Photoaffinity labeling of RPA and XPA by DNA duplexes bearing a photoreactive FAB-dUMP residue at the 5′-end of the primer. The reaction mixture contained 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 1 μg/μl BSA, 0.1 μM 5′-^{32}P-labeled photoreactive DNA, and 1 μM XPA (lanes *2* and *5*) or RPA (lanes *3* and *6*). The reaction mixtures were incubated for 20 min at 37°C and then UV-irradiated (λ = 334-365 nm) for 30 min. The reaction products were analyzed by SDS-PAGE (10% polyacrylamide gel) with subsequent autoradiography. Lanes: *1-3*) DNA with protruding 3′-end of template; *4-6*) nicked DNA. The position of P^{32} at the 5′-end of the primer is marked by an asterisk.

Earlier it was suggested that RPA does not recognize a damage itself but a distortion of double-helix DNA structure caused by this damage [13]. Incorporation of a photoreactive group via the exocyclic amino group of cytosine, unlike the 5′-position of both cytosine and uracil, prevents formation of the canonical Watson–Crick hydrogen bonds; this can cause partial DNA destabilization and influence efficiency of RPA interactions with these substrates. Recently we have shown that DNA bearing an FAP-dCMP residue at the 3′-end of the nick was processed by a bacterial system of nucleotide excision repair [15]. We compared RPA and XPA interaction with DNA structures bearing at the 3′-end of the nick FAP group incorporated either at the 5-position of uracil (FAP-dUMP) or at the 4-position of cytosine (FAP-dCMP) (Fig. 3, a and b). Structures with the 5′-protruding template strand were used for comparison with the nick in both cases. As shown in Fig. 3a, in the case of FAP-dUMP both RPA and XPA are more efficiently modified by DNA duplexes with the protruding template strand. However, when structures with FAP-dCMP were used (Fig. 3b), XPA preferred the nicked DNA, whereas RPA was more efficiently modified by DNA duplex with the protruding end as observed earlier. It should be noted that using the structures with FAP-dCMP (Fig. 3b), we observed that efficiency of XPA modification increased for the nicked structure as well as for the structure with protruding template strand, that is, XPA is sensitive to the substituent distorting the Watson–Crick pairing.

So, one can say that the secondary structure of DNA and mainly the presence of extended single-stranded parts efficiently interacting with RPA plays a key role in RPA binding to DNA. XPA is likely to recognize not only distortion of the Watson–Crick bonds, but also recognizes a bulky substituent itself.

RPA and XPA are known to be necessary for nucleotide excision repair that includes a stage of DNA duplex unwinding around a lesion [35, 36]. According to the recent NER models, XPA and RPA interact with single-stranded DNA, already unwound and bearing an unexcised damage [6]. That is why it was of interest to study interactions of these factors of repair with analogous DNA structures. For this, we constructed DNA duplexes with several unpaired bases (Fig. 1, structure IX) bearing the perfluoroarylazide group at the 5-position of uracil (FAB-dUMP, Fig. 1). For comparison, proteins were modified by photoreactive single-stranded oligonucleotides and complete DNA duplexes (Fig. 1, structures VII and VIII). The results are presented in Fig. 4. As expected, RPA strictly prefers DNA with single-stranded parts and is not significantly modified by complete DNA

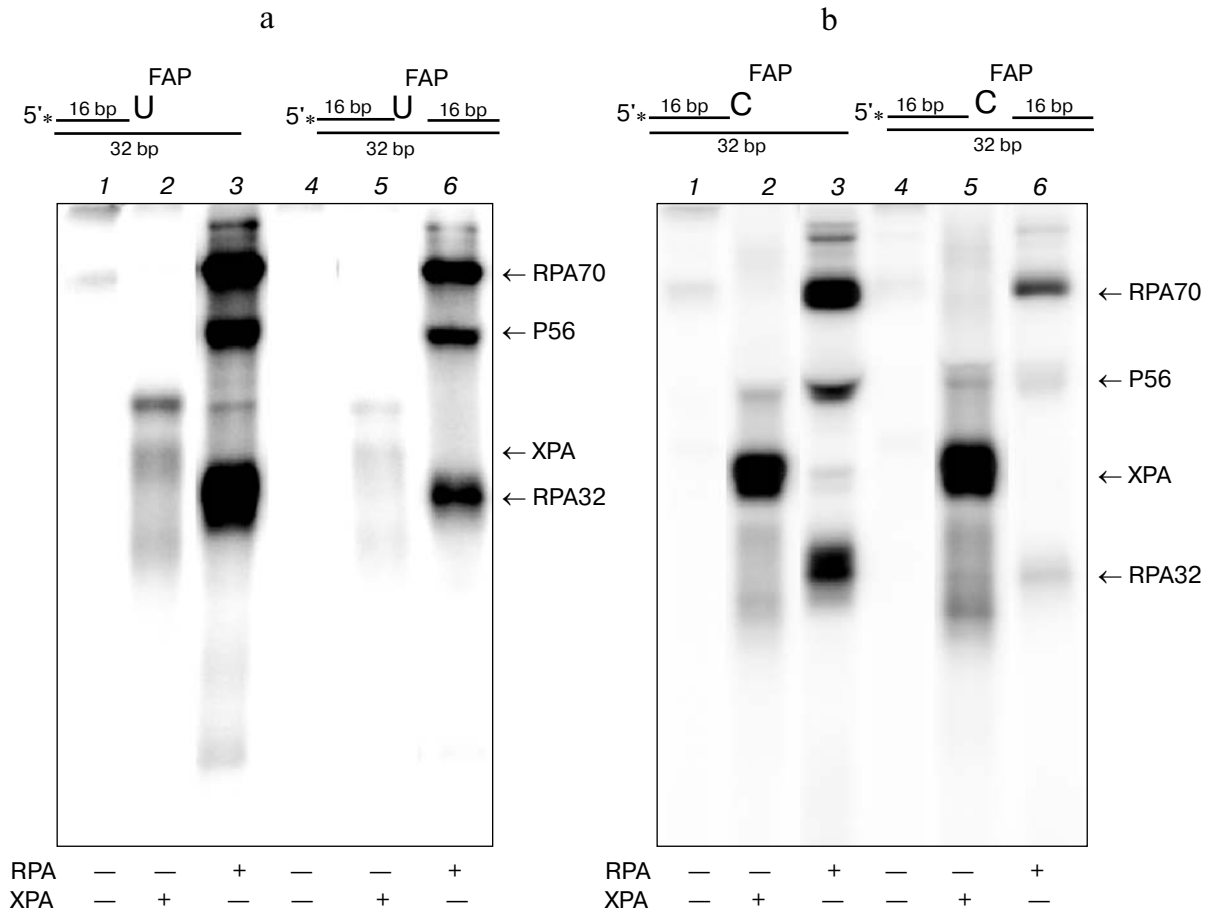


Fig. 3. Photoaffinity labeling of RPA and XPA by DNA duplexes containing photoreactive FAP-dUMP (a) and FAP-dCMP (b) residues at the 3′-end of the primer. The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 1 μg/μl BSA, 0.1 μM 5′-^{32}P-labeled photoreactive DNA, and 1 μM XPA (lanes *2* and *5*) or RPA (lanes *3* and *6*). The reaction mixtures were incubated for 20 min at 37°C and then UV-irradiated (λ = 334-365 nm) for 15 min. The reaction products were analyzed by SDS-PAGE (10% polyacrylamide gel) with subsequent autoradiography. Lanes: *1-3*) DNA with protruding 5′-end of template; *4-6*) nicked DNA. Position of P^{32} at the 5′-end of primer is marked by an asterisk.

duplex. Efficiency of XPA modification is approximately equal for all types of DNA structures used in the experiments. It is likely that it is not the structure of DNA but the presence of a bulky substituent, which plays a key role in DNA recognition by a certain protein. To estimate the effect of a bulky substituent in DNA structure on its interaction with XPA, we compared efficiency of binding of this protein to the native and modified (bearing FAB group inside the strand) single- and double-stranded DNA, using gel retardation. The data show that XPA to some extent prefers to bind to double-stranded DNA rather than to single-stranded DNA; however, significant difference in binding to the native or modified DNA was not revealed (data not presented). It should be noted that available data on efficiency of XPA interaction with single-stranded DNA are contradictory: from efficiency comparable with that for the damaged DNA duplex to that significantly lower (the difference in affinity is several orders of magnitude) [12, 37]. There may be several explanations of the fact that there is no difference in XPA binding to the native and modified DNA in our case. First, FAB-dUMP derivative used does not distort the double helix DNA structure significantly and is not recognized as damage by this protein. Second, gel retardation is not sensitive enough to reveal that these substrates are bound with different efficiency. Finally, the third and most probable reason is that damage recognition is provided not by an individual protein but requires the presence of other NER factors. It is known that XPA affinity to the damaged DNA increases in the presence of RPA [38-40]. We also observed increased XPA labeling in the presence of RPA, the maximal effect being observed for DNA bearing a photoreactive group inside the nick (data not presented).

The data on photoaffinity labeling indicate that XPA obviously prefers the nicked DNA. However, these data may only indirectly account for efficiency of protein interaction with various DNA structures, because effi-

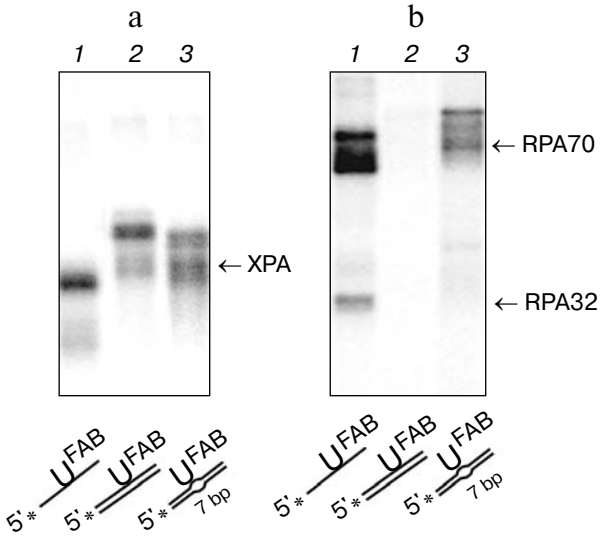


Fig. 4. Photoaffinity labeling of XPA (a) and RPA (b) by DNA structures with a photoreactive FAB-dUMP residue inside the strand. The reaction mixtures contained 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 1 μg/μl BSA, 0.1 μM 5′-^{32}P-labeled photoreactive DNA, and 3 μM XPA (a) or RPA (b). The reaction mixtures were incubated for 20 min at 37°C and then UV-irradiated (λ = 334-365 nm) for 30 min. The reaction products were analyzed by SDS-PAGE (10% polyacrylamide) with subsequent autoradiography. Lanes: *1*) single-stranded DNA; *2*) complete DNA duplex; *3*) DNA duplex with seven non-complementary base pairs. Position of P^{32} at the 5′-end of photoreactive strand is marked by an asterisk.

ciency of labeling depends not only on affinity of complexes. Mutual orientation of photoreactive group and potential protein acceptors, which define efficiency of covalent addition, is also essential. A DNA break may provide additional mobility of the nucleotide neighboring this break, thus enlarging the number of potential target groups available for modification. To evaluate the effect of a nick on XPA and RPA affinity to DNA bearing a bulky photoreactive group, we analyzed protein binding to DNA by gel retardation. Data on XPA (lanes *2-5* and *12-15*) and RPA (lanes *7-10* and *17-20*) binding to DNA bearing FAB-dUMP at the 5′-end of the nick (lanes *1-10*) or inside the strand (lanes *11-20*) are presented in Fig. 5. As shown, XPA binds the nicked DNA more efficiently than a complete duplex, whereas RPA in contrast prefers a complete duplex; this feature is likely to be a consequence of RPA binding to single-stranded DNA (two-fold excess non-modified primer was used at annealing). Since RPA has significantly higher affinity to single-stranded DNA than XPA, complexes with excess non-modified primer are formed first; this lowers the concentration of protein participating in binding to the nicked structure.

Data indicate that the two studied proteins, RPA and XPA, demonstrate different sensitivity to the structures imitating damaged DNA. For RPA, the presence of extended single-stranded parts of DNA plays a key role. RPA is also able to efficiently interact with DNA bearing

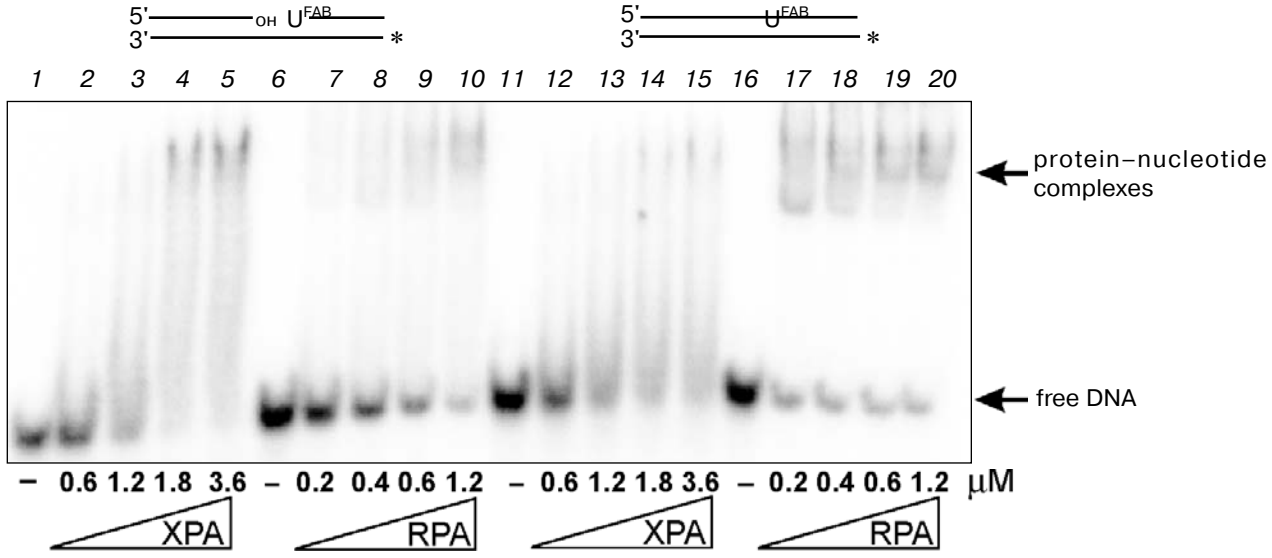


Fig. 5. Binding of XPA and RPA to photoreactive DNA. The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM NaCl, 5% glycerol, 500 μg/ml BSA, 0.01 μM DNA 5′-^{32}P-labeled in the native strand, and various concentrations of XPA (lanes *2-5* and *12-15*) or RPA (lanes *7-10* and *17-20*). The mixtures were incubated for 20 min at 37°C, separated by electrophoresis in 4% native polyacrylamide gel at room temperature, and analyzed by phosphorimager. Lanes: *1-10*) nicked DNA duplex; *11-20*) complete DNA duplex. Position of P^{32} at the 5′-end of the native strand is marked by an asterisk.

a bulky group at the 5′-end of the nick; this indicates possible participation of RPA in recognition of such damages. For XPA, distinct regularities in interaction with DNA substrates were not revealed. This protein seems to have no distinct specificity in interaction with single-stranded and double-stranded DNA, but has affinity to certain types of damages. This is supported by the fact that efficiency of XPA labeling by DNA structures bearing a photoreactive group at the 3′-end of the primer depends on the structure of analog used (FAP-dUMP or FAP-dCMP).

Dimer XPC–hHR23B, another NER protein, has analogous properties; according to recent data, this protein is the most probable factor, which is first to recognize DNA damages [7, 41]. It is also known that XPA affinity to DNA significantly depends on protein–protein interaction with other NER factors, namely RPA [38-40]. XPA is able to influence RPA on binding to certain types of DNA [42].

We suggest that XPA by itself is not able to efficiently recognize damages on the background of large parts of undamaged DNA, but XPA can be a part of a complex in which affinity of any component to DNA damage can increase markedly due to the mutual influence of proteins. XPA either directly participates in recognition and checking of damage, or influences interaction of other proteins. Our suggestion is in accordance with a model of cooperative protein binding to a damage suggested by Reardon and Sancar [43]. According to this model, damage is first detected by any of three proteins—XPA, RPA, or XPC. Cooperative actions of XPA (able to bind RPA and TFIIH), RPA, and XPC (able to bind TFIIH) results in formation of four-component complex on damaged DNA. At the second stage, there occurs kinetic checking of specificity of the formed complex by the helicase activity of TFIIH and termination of reaction in case of formation of nonspecific complex or stimulation of the process in case of formation of specific complex. Such method of scanning for DNA damage is universal and for any type of damage, it allows formation of an efficient complex for repair of this DNA structure.

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