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Interaction of Replication Protein A with Photoreactive DNA Structures

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Abstract—A new photoreactive oligonucleotide derivative was synthesized with a perfluoroarylazido group attached to the 2′-position of the ribose fragment of the 5′-terminal nucleotide. Using this conjugate, photoreactive DNA duplexes were produced which contained single-stranded regions of different length, single-stranded breaks (nicks), and also ds duplex with a photoreactive group inside one of the chains. These structures imitate DNA intermediates generated at different stages of DNA replication and repair. The interaction of replication protein A (RPA) with the resulting DNA structures was studied using photoaffinity modification and gel retardation assay. Independently of the DNA structure, only the large subunit of RPA (p70) was crosslinked to photoreactive DNAs, and the intensity of its labeling increased with decrease in the size of the single-stranded region and was maximal in the case of the nick-containing DNA structure. By gel retardation, the most effective binding of RPA to this structure was shown, whereas the complexing of RPA with DNA containing the unmodified nick and also with the full duplex containing the photoreactive group inside the chain was significantly less effective. The data suggest that RPA should be sensitive to such damages in the double-stranded DNA structure.

Key words: replication protein A, photoaffinity modification, photoreactive oligonucleotides, complex formation

In the eukaryotic cell, the systems of DNA replication and repair responsible for reproduction and maintaining of the genetic information are complicated multicomponent complexes. These complexes include many enzymes and various proteins. The same protein is often involved in several processes and provides a number of important functions. Just such a protein is replicative protein A (RPA), which in eukaryotic cells plays the role of a single-stranded binding protein (SSB-protein). RPA is a stable heterotrimeric complex consisting of subunits with molecular weights of 70, 32, and 14 kD (p70, p32, and p14). The most important biochemical feature of RPA is its high affinity for single-stranded DNA (ssDNA) [1, 2]. A polar binding of RPA to ssDNA was shown, with the highest affinity to the 5′-end of DNA. On binding to a sufficiently long ssDNA, RPA becomes elongated, which results in the polar packing of its DNA-binding domains A, B, C, and D from 5′- to the 3′-end of ssDNA [3-5]. Moreover, RPA can nonspecifically bind to double-stranded DNA (dsDNA) with lower affinity [1, 2]. There are data on specific interaction of RPA with certain sequences of dsDNA which are supposed to be involved in the regulation of transcription [6, 7]. The possibility of specific binding of RPA to dsDNA with a specific structure rather than a certain sequence is also under discussion [8]. The complex formation of RPA with dsDNA damaged by UV or such agents as *cis*-diamminedichloroplatinum (II) was also shown [9-11]. RPA is supposed to be sensitive to modification-caused changes in the structure of dsDNA but bind, as a rule, to the undamaged chain [11]. Consequently, RPA can recognize modifications in dsDNA. But the structure of such complexes is unknown. One of the most common damages of DNA are single-stranded breaks, which are generated, in particular, during base excision repair after removing of a base and by following action of apurinic/apyrimidinic endonuclease-1 (APE). Single-stranded breaks in DNA can also be generated by other mechanisms. Interaction of RPA with nicked dsDNA is poorly understood.

The method of affinity modification [12] has contributed significantly to studies of the interaction of RPA

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with DNA. This method has been used to study the interaction of RPA with DNA duplexes with the protruding ends of the template or gaps of different size [3, 13] and also nicks and flap-structures [14]. A photoreactive aryl-azido group was introduced into the 3′-end of the primer inside various DNA structures using DNA polymerases and base-substituted photoreactive derivatives of dNTP as substrates. DNA structures with a photoreactive group at the 5′-end were produced using an oligonucleotide with perfluoroarylazido group attached to the 5′-terminal phosphate by chemical synthesis [3, 13] that suggested the radiolabeling of the 3′-end of the photoreactive oligonucleotide with DNA polymerase β using [α-^{32}P]dNTP. The present work describes the synthesis of a new photoreactive oligonucleotide analog containing perfluoroarylazido group at the 2′-position of ribose of the 5′-terminal nucleotide residue that allowed us to introduce the radioactive label into the 5′-end of the oligonucleotide using T_4-polynucleotide kinase. This oligonucleotide was used to produce photoreactive DNAs including those with a single-stranded break which imitate intermediates of DNA replication and repair.

An alternative approach, which allowed us to qualitatively and quantitatively analyze the protein complexing with DNA, was gel retardation assay based on changes in the mobility of nucleic acid when complexing with proteins. Combined use of affinity modification and the gel retardation technique is promising for studies of protein–nucleic acid interactions.

MATERIALS AND METHODS

The following reagents were used: [γ-^{32}P]ATP (Biosan, Russia or Amersham, USA); Rainbow markers of molecular weight (Amersham); polynucleotide kinase and DNA ligase of phage T4 (Sibenzyme, Russia); reagents for electrophoresis and main components of buffers (Sigma, USA); 1,4-diaminobutane (FERAX, Germany); $LiClO_4$ (Fluka AG, Switzerland); N-methylimidazole (MeIm) (Sigma); 2,6-dimethylpyridine (Merck, Germany); phosphoramidites, tetrazole (Cruachem, Scotland). Other reagents and solvents were of domestic production, special purity. Organic solvents N,N-dimethylformamide (DMF), triethylamine (TEA), acetone, acetonitrile (MeCN), pyridine, monoethanolamine, and acetic anhydride were purified and dried by standard procedures [15].

Oligonucleotides used in the work were synthesized by Genset (France) or in the Novosibirsk Institute of Bioorganic Chemistry using an ASM-800 oligonucleotide synthesizer (BIOSSET, Russia) and desalted with an RP-cartridge (Cruachem) on an OPS-201 system for oligonucleotide purification (BIOSSET). Preparative HPLC was performed using a Waters 600 chromatograph supplemented with a Waters 486 UV-detector on a 0.78 × 30 cm column with LiChroprep RP-18, 15-25 μm (Merck, USA), gradient from 0 to 80% MeCN in 0.03 M $LiClO_4$ for 60 min, and rate of elution 6 ml/min.

Recombinant RPA was isolated from a strain of *E. coli* according to [16]. Recombinant DNA polymerase β, flap-endonuclease-1, apurinic/apyrimidinic endonuclease-1, and poly(ADP-ribose) polymerase-1 were isolated from *E. coli* strains by standard procedures [17-19], respectively, and were kindly presented by S. N. Khodyreva.

Synthesis of an oligonucleotide derivative containing the aminobutane linker was performed by the standard solid phase phosphoramidite procedure. At the last stage, 3-phosphoramidite of modified uridine (Fig. 1) presynthesized according to [20, 21] was attached to the oligonucleotide. Every 5 min 6 μl-portions of the modified phosphoramidite and tetrazole were pumped throughout the reactor. Condensation was performed at 30°C for 30 min. The synthesized oligonucleotide attached to the solid phase carrier was subsequently treated with 1,4-diaminobutane for 2 h at 60°C. Then the polymer was supplemented with solution of monoethanolamine–water (2 : 1) and maintained for 1 h at 60°C in an air thermostat. The oligonucleotide was precipitated with $LiClO_4$ in acetone, washed with acetone and ether. An oligonucleotide 5′-GT-ACG-GAT-CCC-CGG-GTA-C-3′ was synthesized as control by the standard solid phase phosphoramidite procedure.

Photoreactive group was attached to the oligonucleotide, which contained the aminobutyl linker group as follows. The oligonucleotide was dissolved in 5 μl of water, diluted with 50 μl of DMF, and the solution was supplemented with 50 μl of DMF containing 3.3 mg (10 μmol) N-hydroxysuccinimide ester of *p*-azidotetrafluorobenzoic acid and 7 μl (50 μmol) triethylamine. The reaction mixture was maintained with shaking for 2 h at room temperature, and then the oligonucleotide conjugate was precipitated with 6% $LiClO_4$ in acetone and washed with acetone and ether. Purity of the resulting conjugate was monitored by HPLC.

^{32}P-Label at the 5′-end of the primer was introduced with T4 polynucleotide kinase as described in [22]. The reaction mixture (20 μl) contained 1 μM primer, [γ-^{32}P]ATP, T4 polynucleotide kinase (5 activity units). The reaction was performed for 30 min at 37°C and maintained overnight at 4°C. Then the mixture was separated by electrophoresis in denaturing polyacrylamide gel as described in [23]. The nucleotide substance was electroeluted onto a DE-81 paper from the gel region located by radioautography. As electrode buffer Tris-borate buffer (50 mM, pH 8.3) was used. The product was eluted from DE-81 with three 20-μl portions of hot 3 M $LiClO_4$. The eluate was supplemented with 1.2 ml of acetone cooled to 4°C, and the mixture was maintained at –40°C for 1 h. The precipitate was centrifuged, washed twice with 1 ml

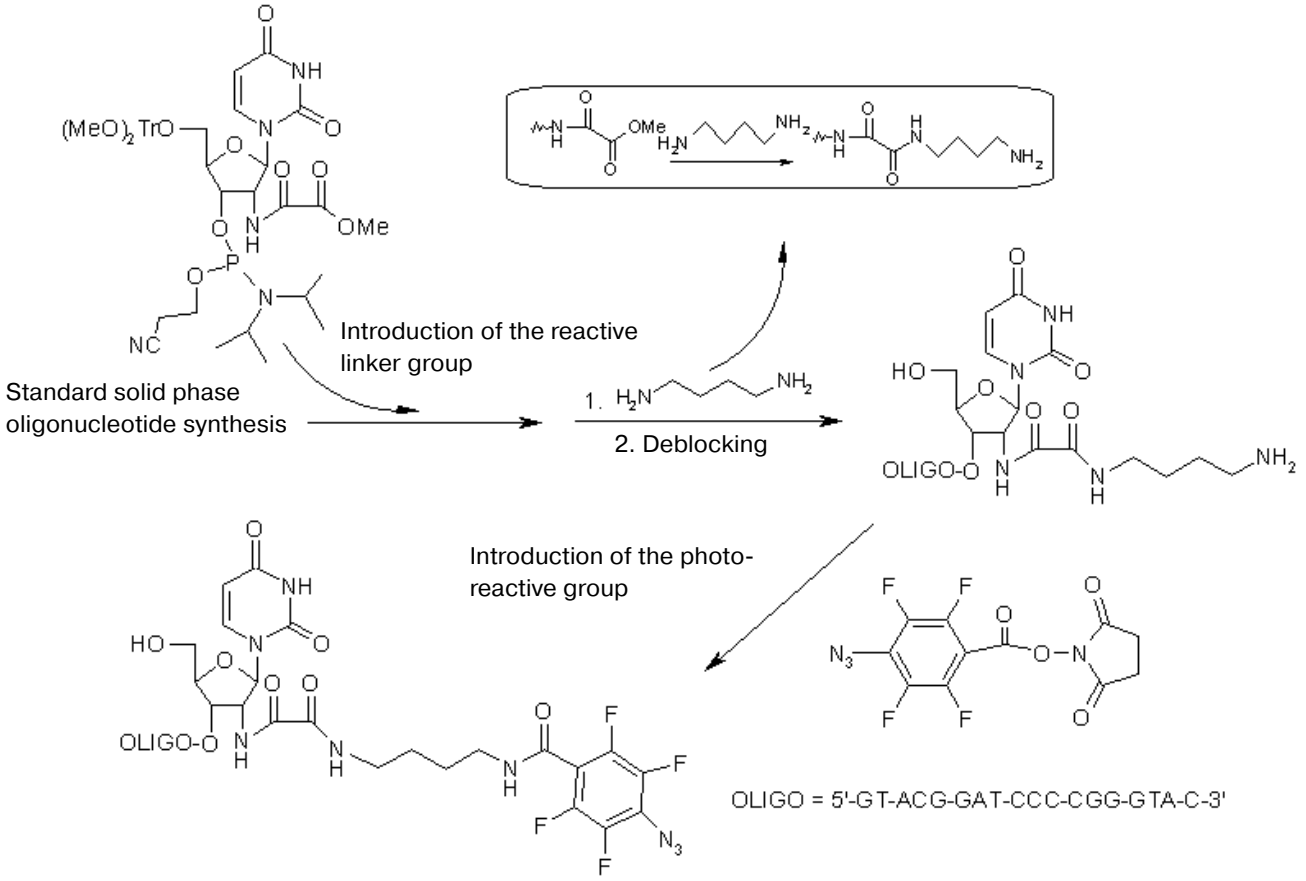

Fig. 1. Scheme of synthesis of photoreactive oligonucleotide derivative.

of acetone cooled to 4°C, dried, and then diluted in water to the required concentration.

Photoaffinity modification of RPA was performed in 10 μl of reaction mixture, which contained standard buffer components (50 mM Tris-HCl, pH 7.8 (at 25°C), 50 mM NaCl, 5 mM $MgCl_2$), 1 μM 5′-^{32}P-labeled photoreactive complex of primer-template, and 1 μM RPA, at 37°C for 20 min. The reaction mixtures were UV-irradiated for 20 min using an HBO W high-pressure mercury lamp with a Bausch and Lomb monochromator in the region of 320 nm. The modification products were analyzed by SDS electrophoresis in polyacrylamide gel with subsequent radioautography.

The oligonucleotides were ligated by addition of T4 DNA ligase (20 activity units) (the activity unit corresponded to the enzyme quantity required for 50% cross-linking of *Hind*III-fragments of phage λ DNA in 30 min at 16°C in 20 μl of the reaction mixture containing 300 μg/ml DNA) and ATP (1 mM final concentration) to 10 μl of the mixture. The reaction was performed for 1.5 h at room temperature. The cross-linked oligonucleotide was isolated by electrophoresis in denaturing polyacrylamide gel. Thus, the ^{32}P-labeled oligonucleotide was produced with a photoreactive group in the internal region of the chain.

Electrophoresis of protein–nucleic acid complexes under native conditions was performed at 4°C in 6% polyacrylamide gel (50 mM Tris-OAc, pH 8.3, 6% acrylamide, 0.2% bisacrylamide, 50 mM $Mg(OAc)_2$, 0.1% TEMED, 0.05% $(NH_4)_2S_2O_8$). Before placing onto the gel, the specimens to be separated were supplemented with 1/5 volume of buffer which contained 25% glycerol and 0.05% Bromophenol Blue. Electrophoresis was performed in vertical plates of 12 × 10 × 0.1 cm, with 50 mM Tris-OAc (pH 8.3), 5 mM $Mg(OAc)_2$ as electrode buffer. Samples were placed onto the gel at 200 V, and electrophoresis was performed at 10 V/cm until the half-way migration of Bromophenol Blue. The radioactively labeled protein–nucleic acid complexes were identified by radioautography.

Association constants of RPA with different DNA structures were determined by varying the protein concentration. The reaction was performed in 10 μl of reaction mixture, which contained 0.01 μM 5′-^{32}P-labeled DNA, various concentrations of RPA (0.1-8 μM), and also standard buffer components. To determine the fraction of the

Structure 1

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5′-GTACCCGGGGATCCGTACGGCGCATCAGCTGCTAAGTTCTAGTGTATAC-3′
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Structure 2

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5′-GTACCCGGGGATCCGTACGGCGCATCAGCTGCTAAGTTCTAGTGTATAC-3′
3′-CATGGGCCCCTAGGCATGU^R-5′
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Structure 3

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5′-GTACCCGGGGATCCGTACGGCGCATCAGCTGCTAAGTTCTAGTGTATAC-3′
3′-CATGGGCCCCTAGGCATGU^R-5′  AGTCGACGATTCAAGATCACATATG-5′
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Structure 4

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5′-GTACCCGGGGATCCGTACAGCGCATCAGCTGCAG-3′
3′-CATGGGCCCCTAGGCATGU^R
                    3′-CGCGTAGTCGACGTC-5′
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Structure 5

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5′-GTACCCGGGGATCCGTACAGCGCATCAGCTGCAG-3′
3′-CATGGGCCCCTAGGCATG
                   3′-TCGCGTAGTCGACGTC-5′
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Structure 6

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5′-GTACCCGGGGATCCGTACAGCGCATCAGCTGCAG-3′
3′-CATGGGCCCCTAGGCATGU^RCGCGTAGTCGACGTC-5′
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Structure 7

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5′-GTACCCGGGGATCCGTACAGCGCATCAGCTGCAG-3′
3′-CATGGGCCCCTAGGCATGUCGCGTAGTCGACGTC-5′
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Fig. 2. Structures of oligonucleotide complexes. U^R is the modified uridine residue (2′-carbamoyl deoxyuridine).

active RPA, the concentration of single-stranded DNA was varied. Reaction mixtures (10 μl) containing 0.5 μM RPA, various concentrations of 5′-^{32}P-labeled DNA (0.001-0.5 μM), and also standard buffer components were incubated for 20 min at 37°C and analyzed by electrophoresis under the native conditions. Radiolabeled oligonucleotides and protein-nucleic acid complexes were located by radioautography, then from the polyacrylamide gel strips which corresponded to the free and bound DNA substrate were cut off and counted using a Cherenkov scintillation counter. The data were processed using the Microcal Origin 5.0 Program (Microcal Software, USA).

RESULTS AND DISCUSSION

Oligonucleotide conjugate containing a photoreactive 2′-carbamoyl group attached to the 5′-terminal link of the oligonucleotide was synthesized using activated spacer groups [20, 21]. Figure 1 presents the scheme of the synthesis. At the last stage of the standard solid phase oligonucleotide synthesis, into the reaction of condensation was introduced phosphoramidite, which contained a methoxyoxalylamide group in the 2′-position of the ribose fragment of the nucleoside. To attach the modified monomer, the standard scheme of synthesis was used, with the time of condensation increased to 30 min. Prior to deblocking and removal from the resin, the oligonucleotide was treated with 1,4-diaminobutane. The oligonucleotide conjugate containing an aminobutyl linker group in the 2′-position of the 5′-terminal nucleotide moiety was isolated as described in "Materials and Methods". The photoreactive group was introduced into the oligonucleotide using N-hydroxysuccinimide ester of tetrafluoroazidobenzoic acid [24]. Into the oligonucleotide containing the aminobutyl linker, a photoreactive group was introduced by treatment of the oligonucleotide conjugate with an excess of N-hydroxysuccinimide ester of *p*-tetrafluoroazidobenzoic acid. HPLC analysis showed that the purity of the resulting oligonucleotide conjugate was no less than 95%.

The resulting photoreactive oligonucleotide conjugate with perfluoroarylazido group in the 2′-position of the 5′-terminal uridine was used to model DNA structures generated during different stages of long-patched and short-patched excision repair of bases. Such structures can also appear during replication of DNA. Structures of oligonucleotide complexes are shown in Fig. 2. To determine the interaction with these structures of possible participants of the DNA repair, we studied photoaffinity modification of a number of purified proteins (at comparable concentrations): DNA polymerase β, flap-endonuclease-1, apurinic/apyrimidinic endonuclease-1, poly(ADP-ribose) polymerase-1, replication protein A. Among these proteins, replication protein A and DNA polymerase β interacted with these structures most efficiently and with a low efficiency, respectively. Afterwards we studied only the interaction of RPA with the above-listed DNA structures.

Replication protein A may be considered to be a protein involved in the replication of DNA, repair of nucleotides [25], and also repair of bases [26]. However, the last function of RPA is insufficiently studied. It was interesting to study the interaction of RPA with DNA structures which have the photoreactive 5′-end of the primer turned to the protruding 3′-end of the template and also with DNA duplexes containing gaps of different length (15, 5, and 0 nucleotides). Figure 3 presents results of electrophoretic separation of products of the RPA crosslinking with photoreactive DNA duplexes. The data

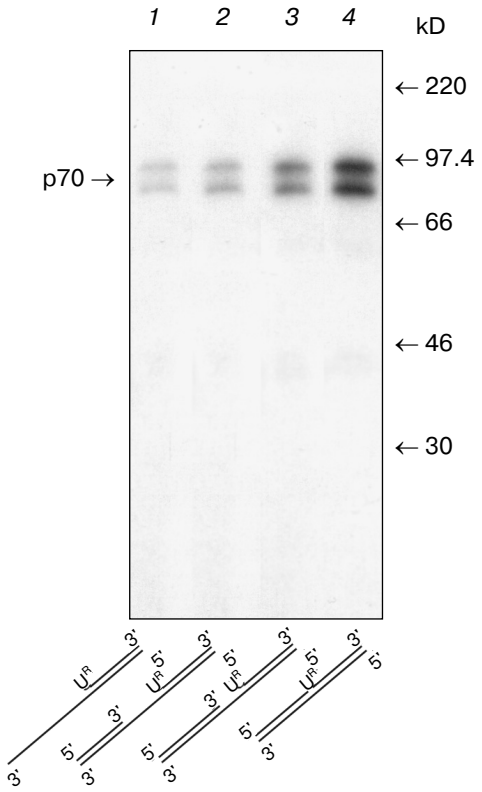

Fig. 3. Photoaffinity modification of RPA with photoreactive DNA. 5′-^{32}P-labeled DNA duplex was incubated in the presence of RPA for 20 min, then the reaction mixtures were UV-irradiated at 320 nm. The modification products were separated by 12% SDS-PAGE with subsequent radioautography. The molecular weights of the proteins are presented in kD on the right.

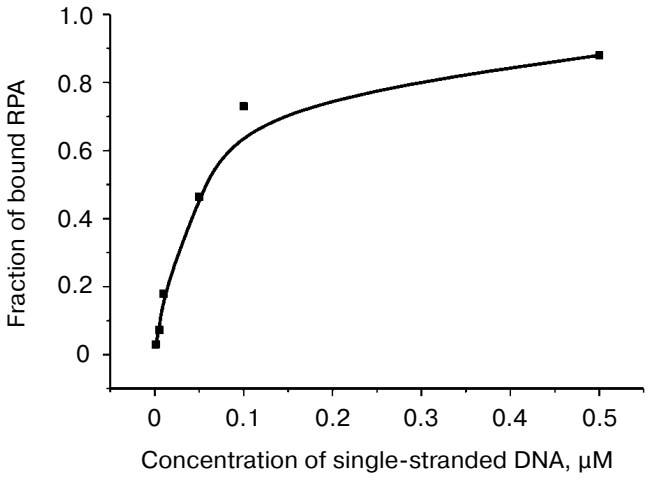

Fig. 4. Determination of the active protein fraction in the preparation of RPA.

suggested that all structures produced the labeled p70 subunit of RPA, along with generation of two products of photoattachment to this polypeptide, and this fact is in agreement with the previous data [3, 13]. Intensity of the labeling increased with decrease in the gap size suggesting the effective interaction of RPA with this structure. At RPA concentrations higher than the concentration of DNA, the labeling type of the protein did not change, and only the large subunit of RPA (p70) was modified. Data on affinity modification can only indirectly suggest the difference in the effectiveness of interaction of a biopolymer with the various structures of photoaffinity reagent. A significant role can often be played by the mutual orientation of the affinity reactive group and biopolymer, which changes the direction of the covalent binding. This could also be significant in the case under consideration. To explain the observed regularities, we determined by gel retardation the affinity of RPA for the above-mentioned DNA structures. This approach allowed us to estimate the order of the association constant values of protein-nucleic acid complexes and also detect possible cooperative interactions.

To determine K_{ass} values for the interaction of RPA with DNA structures, it was necessary to determine the amount of active protein in the RPA preparation under study. Aliquots of RPA were incubated with the various amounts of 5′-^{32}P-labeled ssDNA (structure 1, Fig. 2). Figure 4 presents the dependence of the active protein fraction on concentration of ssDNA. The figure shows that the maximum RPA bound in the complex is 88% of the total amount of the protein in the preparation. Thus, the RPA preparation has high DNA-binding activity.

For the control, parameters of RPA binding to 5′-^{32}P-labeled ssDNA (structure 1) were determined. Aliquots of 5′-^{32}P-labeled ssDNA were incubated with the different amounts of RPA. The complexes were separated in 6% polyacrylamide gel (data not presented). Two kinds of complexes were detected. An increase in the concentration of RPA resulted in the increase in the fraction of less mobile complexes, which corresponded to the binding to DNA of two RPA molecules. This fact is in agreement with the literature data on the positive cooperation in the binding of RPA to the elongated single-stranded DNA [6].

Association constants of RPA with DNA were similarly determined in the case of DNA structures 2-5. To compare the effect of modification of the sugar residue in the case of dsDNA structure and DNA with single-stranded break, we have also studied the binding of RPA to dsDNA, which contained either modified or native uridine residue (structures 6 or 7, respectively). The photoreactive group was introduced into dsDNA by DNA ligase of phage T4 catalyzed generation of the phosphodiester bond in the nick with uridine derivative modified in the 2′-position of ribose at the 5′-end of the nick. The association constants of RPA–DNA complexes were calculated from the Langmuir's equation for the reaction of bimolecular binding [27]. The association constant values for the complexes studied are presented in the table, and experimental data used for their calculation are shown in Fig. 5.

The table shows that RPA displayed the highest affinity for DNA structures 1, 2, and 4. In the case of structures 1 and 2, this was due to the main function of RPA, the binding of single-stranded DNA. Note that two kinds of complexes were observed for both single-stranded DNA and DNA with the protruding 3′-end of the template, and this was likely to be caused by the presence of a rather elongated single-stranded region (30 bp) sufficient for binding two RPA molecules (Fig. 5a). No multiple binding was, as a rule, displayed by RPA on binding to structures lacking an elongated single-stranded region (Figs. 5b-5d). The table shows that the affinity of RPA for structure 4 with the modification at the 5′-end of the nick was significantly higher than for the other structures without elongated single-stranded regions. The presence of unmodified nick (structure 5) and also modification of the sugar residue in the absence of the chain break (structure 7) only insignificantly influenced the RPA affinity compared to the native duplex 6. However, the interaction of RPA with DNA containing a modified nucleoside at the 5′-end of the nick was nearly an order of magnitude more effective than with a similar structure without modification. Thus, these data suggested that RPA should be sensitive to breaks in the double-stranded structure of

Association constants of RPA complexes with DNA structures

DNA structure	$K_{ass} \times 10^{-7}$, M^{-1}
Structure 1 (single-stranded DNA of 49 bp in length)	6.2 ± 0.3
Structure 2 (double-stranded DNA with the 3′-protruding end of 30 bp in length)	1.4 ± 0.6
Structure 3 (gap of 5 bp in length with the modified uridine at the 5′-end)	0.13 ± 0.03
Structure 4 (nick with the modified uridine at the 5′-end)	1.5 ± 0.5
Structure 5 (nick with unmodified nucleotide at the 5′-end)	0.23 ± 0.04
Structure 6 (double-stranded DNA of 34 bp in length)	0.14 ± 0.03
Structure 7 (double-stranded DNA of 34 bp in length with the modified uridine)	0.3 ± 0.09

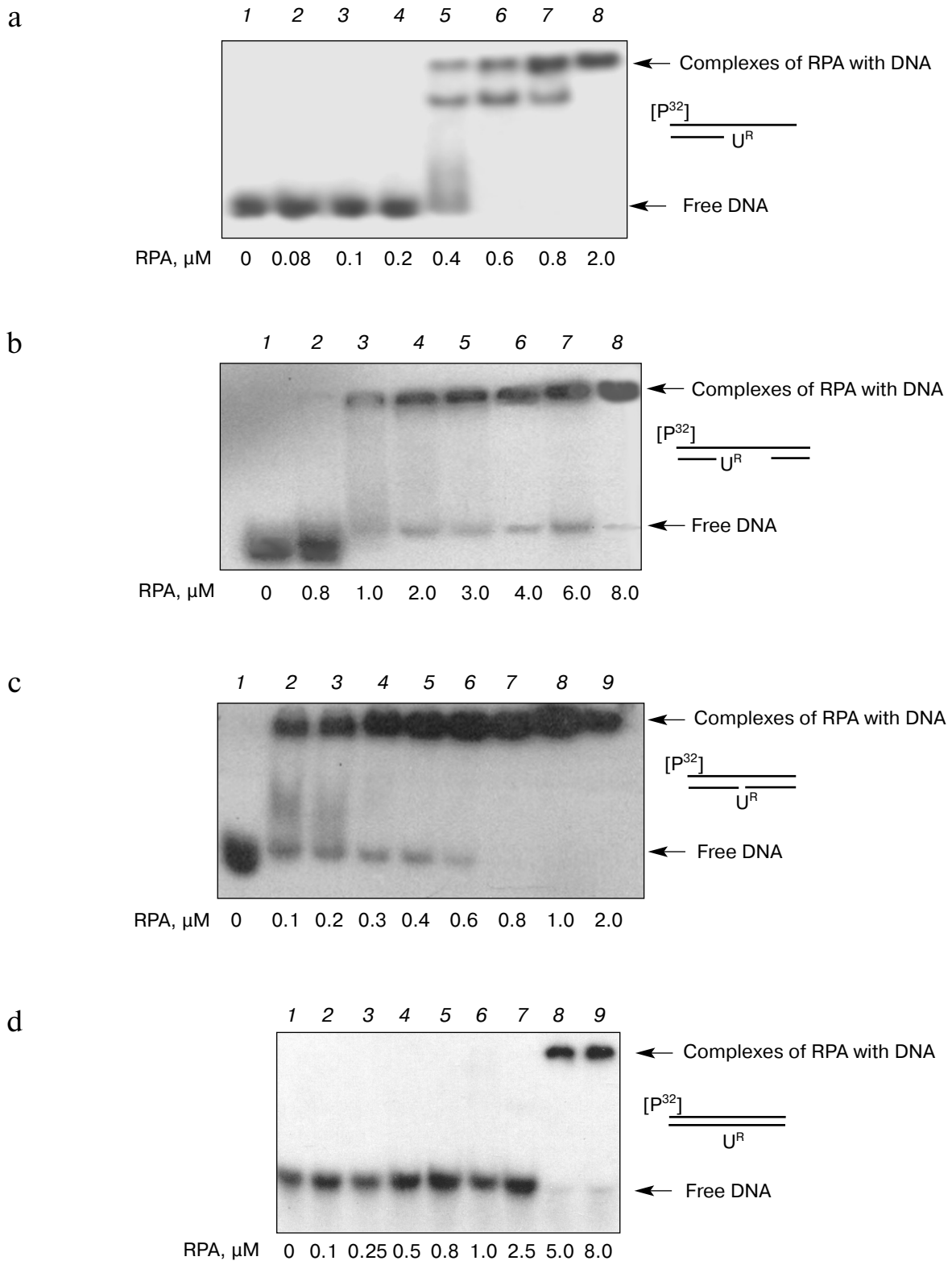

Fig. 5. Analysis by gel retardation of RPA complexes with the different DNAs: a) DNA duplex containing the protruded 3′-end; b) DNA duplex containing a gap of 5 bp in length with the modified uridine at the 5′-end; c) DNA duplex containing a nick with the modified uridine at the 5′-end; d) DNA duplex containing modified uridine inside the chain. Lanes: *1*) control mixtures without addition of RPA; *2-9*) varied concentrations of RPA. The complexes were separated in 6% polyacrylamide gel under native conditions with subsequent radioautography.

DNA in the presence of a modified moiety at the 5′-end of the break. The high affinity of RPA to structure 4 was likely to be due to the presence of a rather bulky substituent at the 5′-end of the primer. RPA seemed to recognize this DNA structure as a damaged DNA because K_{ass} value for structure 5 was virtually by an order of magnitude lower. It is interesting that effectiveness of the interaction of RPA with modified DNA was higher in the presence of a break. It is suggested that the presence of a break near the modified ribose residue should increase the probability of RPA binding to the complementary undamaged DNA chain [11]. This is consistent with some hypotheses on the "sensitivity" of RPA to damages in DNA that most likely suggests the involvement of replication protein A in various DNA repair processes as a lesion sensor [28-33].

The different effectiveness of photoaffinity modification of RPA by structures containing an elongated region of single-stranded template compared to those with gaps and nicks seems unlikely to be due only to difference in the RPA affinity for these structures. Changes in the RPA conformation seem also to play an important role in its binding to different DNA structures, and this, in turn, can result in different mutual orientation of protein acceptors and reactive residue of DNA.

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