602–607 Nucleic Acids Research, 1998, Vol. 26, No. 2

Subunits of human replication protein A are crosslinked by photoreactive primers synthesized by DNA polymerases

Olga I. Lavrik[1,*], Heinz-Peter Nasheuer[2], Klaus Weisshart[2], Marc S. Wold[3], Rajendra Prasad[4], William A. Beard[4], Samuel H. Wilson[4] and Alain Favre

Institut Jacques Monod CNRS, 75251 Paris Cedex 05, France, [1]Novosibirsk Institute of Bioorganic Chemistry Siberian Division of Russian Academy of Sciences, 630090 Novosibirsk, Russia, [2]Institut für Molekulare Biotechnologie Jena, D-07708 Jena, Germany, [3]University of Iowa School of Medicine, Iowa City, IA 52242-1109, USA and [4]National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA

Received August 26, 1997; Revised and Accepted November 7, 1997

ABSTRACT

Human replication protein A (huRPA) is a multisubunit protein which is involved in DNA replication, repair and recombination processes. It exists as a stable heterotrimer consisting of p70, p32 and p14 subunits. To understand the contribution of huRPA subunits to DNA binding we applied the photoaffinity labeling technique. The photoreactive oligonucleotide was synthesized *in situ* by DNA polymerases. 5-[*N*-(2-nitro-5-azidobenzoyl)-*trans*-3-aminopropenyl-1]deoxyuridine-5′-triphosphate (NABdUTP) was used as substrate for elongation of a radiolabeled primer√template either by human DNA polymerase α primase (polα), human DNA polymerase β (polβ) or Klenow fragment of *Escherichia coli* DNA polymerase I (KF). The polymerase was incubated with NABdUTP and radiolabeled primer–template in the presence or absence of huRPA. The reaction mixtures were then irradiated with monochromatic UV light (315 nm) and the crosslinked products were separated by SDS–PAGE. The results clearly demonstrate crosslinking of the huRPA p70 and p32 subunits with DNA. The p70 subunit appears to bind to the single-stranded part of the DNA duplex, the p32 subunit locates near the 3′-end of the primer, while the p14 subunit locates relatively far from the 3′-end of the primer. This approach opens new possibilities for analysis of huRPA loading on DNA in the course of DNA replication and DNA repair.

INTRODUCTION

The eukaryotic single-stranded DNA (ssDNA) binding protein replication protein A (RPA) plays an important role in DNA replication, nucleotide excision repair and homologous recombination. RPA stabilizes the denatured configuration of DNA and makes specific contacts with other proteins involved in DNA replication, repair and recombination. Replication functions of RPA have been extensively studied using DNA replication systems *in vitro* (1–3). Briefly, human RPA (huRPA) interacts with the simian virus 40 large T antigen–origin initiation complex to stimulate origin denaturation and interacts specifically with T antigen and the host DNA polymerase α primase (polα) by forming a specific complex that is required for primer synthesis (4–8). Stimulation of activity of DNA polymerases α, δ and ε by huRPA has also been demonstrated (8–12).

Eukaryotic huRPA is a stable heterotrimer consisting of three subunits with apparent molecular masses of 70, 32 and 14 kDa, termed p70, p32 and p14 respectively (1,13,14). It was suggested that the p70 subunit is primarily responsible for binding to ssDNA (15,16), while the p32 subunit is a target for cell cycle-dependent phosphorylation (17–19) and presumably contributes to binding of huRPA to ssDNA (20). Subunit p32 forms a stable complex with the p14 subunit and that complex tightly binds to the p70 subunit (21,22). The role of the p14 subunit is less clear, though it is necessary for complete assembly of a functional form of RPA (15).

Investigation of the interaction of various huRPA subunits with DNA and their interactions with other components of the DNA replicative machinery seems to be essential. Numerous base-substituted dNTP analogs conjugated to arylazido groups, via linkers, were synthesized and characterized for their ability to be used as substrates of different DNA polymerases (23–27). Photochemical characteristics of these analogs allow UV irradiation of the reaction mixtures by near-UV light to be carried out without photochemical damage to proteins and nucleic acids. These analogs were applied for ‘catalytically’ competent labeling of the active sites of human polα (24), human DNA polymerase β (polβ) (25) and human immunodeficiency virus reverse transcriptase (HIV-RT) (25–27).

Since the function of the two smaller subunits of the heterotrimeric RPA complex is still obscure, we decided to use a base-substituted photoreactive derivative of deoxyuridine 5′-triphosphate for synthesis of photoreactive primer *in situ* using DNA polymerases. A template–primer system was designed containing a single-

*To whom correspondence should be addressed. Tel: +7 383 235 0296; Fax: +7 383 235 1665; Email: lavrik@modul.bioch.nsk.su

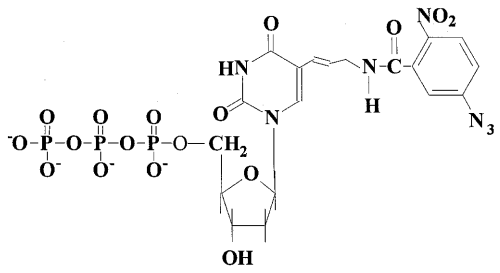

Figure 1. Structural formula of the photoreactive dNTP analog NABdUTP.

stranded template tail that was long enough to bind huRPA. We determined that the single-stranded tail is required for cross-linking of huRPA to a photoreactive primer containing an arylazido group at the 3′-end. The arrangement of huRPA subunits bound to DNA was examined using this approach.

MATERIALS AND METHODS

Recombinant polα was expressed using baculovirus vectors and purified as described (28–30). Recombinant polβ and Klenow fragment of *Escherichia coli* DNA polymerase I (KF) were purified according to Beard and Wilson (31) and Joyce and Grindley (32) respectively. huRPA was expressed in *E.coli* and purified as described (33,34). Bovine serum albumin (BSA) was from Sigma. Rainbow colored protein molecular mass markers were from Amersham. Prestained SDS–PAGE molecular mass markers were from Sigma. T_4 polynucleotide kinase was from New England Biolabs. [γ-^{32}P]ATP was from ICN. Synthetic oligonucleotides were obtained from GENSET or synthesized in the Institut Jacques Monod (Paris). Nensorb-20 columns were from Du Pont. NABdUTP (Fig. 1) was synthesized and characterized as described (23). Polyclonal antibodies against huRPA that preferentially recognize p32 and to lesser extents p70 and p14 were produced as described (35).

Radioactive labeling of oligonucleotide primers

Dephosphorylated primers were 5′-phosphorylated with T_4 polynucleotide kinase as described (36). Unreacted [γ-^{32}P]ATP was separated by passing the mixture over a Nensorb-20 column using the manufacturer's suggested protocol.

Primer–template annealing

Lyophilized oligonucleotides were resuspended in 10 mM Tris–HCl, pH 7.4, and 1 mM EDTA and the concentrations determined from their UV absorbence at 260 nm. Primers–templates were annealed at a molar ratio of 1:1 by heating at 90°C for 3 min and then the mixture was allowed to cool down slowly to room temperature. The sequence of the primers and templates used were as follows:

```
system 1   5′-GGTTAAATAAAATAGTAAGAATGTATAGCCCCTACC-3′
                                3′-TACATATCGGGGATGG-5′

system 2   5′-AATGTATAGCCCCTACC-3′
             3′-TACATATCGGGGATGG-5′
```

Photochemical crosslinking

huRPA was photoaffinity labeled with photoreactive primer synthesized *in situ* using NABdUTP and polα, polβ or KF. Reaction mixtures (20 µl) contained 50 mM Tris–HCl, pH 7.8, 10 mM $MgCl_2$, 50 mM KCl, 0.5–10 µM polymerase, 0.5 µM template·5′-[^{32}P]primer, 40–50 µM NABdUTP and huRPA to a final concentration of 0.1–0.5 µM, as indicated. The reaction mixtures were incubated at 25°C for 30–45 min to allow elongation of the primers. Then the mixtures were spotted on Parafilm that was placed on ice and UV irradiated with a Baush and Lomb monochromator equipped with a HBO W super pressure mercury lamp producing UV light of 315 nm for 15 min. Reactions were stopped by adding Laemmli buffer and heating. The photochemically crosslinked protein–DNA samples were separated by SDS–PAGE. Dried gels were subjected to autoradiography or quantified using a phosphorimager (Molecular Dynamics). Immunoprecipitation procedures were according to *Current Protocols in Molecular Biology*, Suppl. 21 (35).

DNA polymerization assay

Enzyme activities of polα, polβ and KF were determined using a standard protocol in a reaction volume of 50 µl containing 50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$ and 50 mM KCl, template–primer, enzyme and [α-^{32}P]dNTP. Reactions were started by addition of enzyme, incubated at 25°C for the indicated time intervals and stopped with 20 µl 0.5 M EDTA, pH 8.0. Quenched reaction mixtures were spotted onto Whatman DE-81 filter disks and dried. Unincorporated [α-^{32}P]dNTP was removed and filters were counted as described (37).

RESULTS

Substrate and photochemical properties of NABdUTP

The special photochemical properties of the photoreactive base-substituted dUTP analog NABdUTP allow UV activation to be performed by near-UV light, i.e. >300 nm, which is far beyond the absorbance intensities of nucleic acids and proteins. In particular, the reagent has been found to be very effective for modification of proteins. In contrast, NABdUTP has no DNA crosslinking activity, which allows internal crosslinking of DNA duplexes to be avoided when NABdUMP is incorporated into the primer (27).

Earlier we have shown that NABdUTP is a good substrate for HIV-RT (27), mammalian polα, human polβ and KF (data not shown). This property of the analog was utilized for incorporation of the photoreactive dUMP moiety into the 3′-end of a 5′-^{32}P-labeled primer. Synthesis of photoreactive primer was carried out *in situ* prior to UV irradiation. The products of UV crosslinking were separated by SDS–PAGE and visualized by autoradiography. The primer containing the incorporated dUMP analog can be efficiently crosslinked with polβ and KF (Fig. 2, lanes 5 and 8), while the primers without the dUMP analog do not exhibit any labeling of polymerases under similar experimental conditions (Fig. 2, lanes 4 and 7). We did not detect any significant labeling of polα, due to the low efficiency of labeling at the protein concentrations used (Fig 2, lane 2). However, longer exposure of the gel permits labeling of polα core polypeptide (~180 kDa) to be revealed (data not shown).

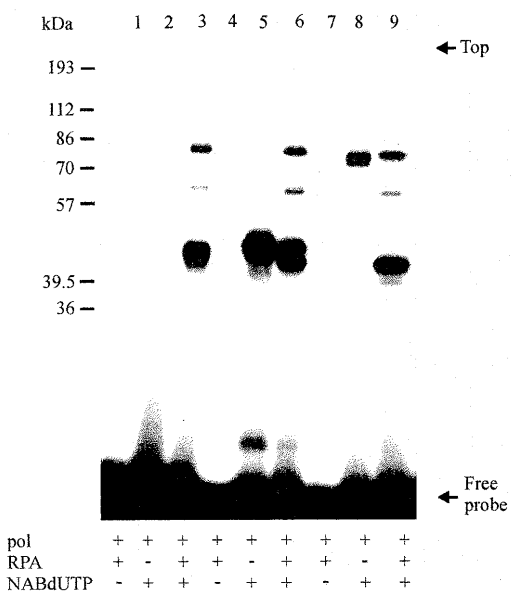

Figure 2. Photoaffinity labeling of huRPA by photoreactive primers. To investigate labeling of huRPA the reaction mixtures were composed of 0.56 µM template–primer (system 1), 40 µM NABdUTP, 0.64 µM huRPA and different DNA polymerases, as indicated. Lanes 1–3, reaction mixtures contained template–primer and 0.4 µM recombinant polα; in addition, lanes 1 and 3 had huRPA and lanes 2 and 3 contained NABdUTP. Lanes 4–6, template–primer and 5 µM polβ were used. Additional components were huRPA (lanes 4 and 6) and for NABdUTP (lanes 5 and 6). Lanes 7–9, template–primer and 1 µM Klenow fragment of *E.coli* DNA polymerase I (KF) were incubated together with huRPA (lanes 7 and 9) and NABdUTP (lanes 8 and 9). Reaction mixtures were incubated for 40 min at 25°C and then UV irradiated for 15 min ($\lambda_{315\,nm}$). The crosslinked protein–DNA complexes were separated by SDS–PAGE and visualized by autoradiography. The positions of the free probe and the protein markers are indicated.

Photoaffinity labeling of huRPA by photoreactive DNA

NABdUMP was incorporated into ^{32}P-labeled template–primer (system 1; see Materials and Methods) either in the absence or presence of huRPA. To avoid possible DNA unwinding activity, which is stimulated by huRPA at low ionic strengths (38,39), all reactions were carried out under high salt conditions. In the experiments UV irradiation itself does not influence the specific activity of the DNA polymerases used (data not shown). Under similar conditions, as depicted in Figure 2, lane 2, addition of huRPA results in crosslinking of the huRPA p70 subunit, its proteolytic fragment and the p32 subunit to the photoreactive [^{32}P]primer–template (Fig 2, lane 3). A band corresponding to the product of highest molecular weight, i.e. 78 kDa, arises from the crosslinking product of the primer with the p70 subunit. A band corresponding to the product of 62 kDa appears to be a crosslinking product of the primer with a proteolytic fragment generated from p70. The fastest migrating radiolabeled product most likely corresponds to the p32 subunit covalently crosslinked to the radiolabeled 17mer primer. It is worth noting that each determined product (protein covalently bound to primer) is retarded by a value of ~6–8 kDa relative to that of the unmodified protein.

In order to demonstrate that the crosslinking property of huRPA does not depend on the type of DNA polymerase, affinity labeling of huRPA was performed with polβ and KF (Fig. 2, lanes 4–9). In the absence of huRPA only polβ is clearly crosslinked (Fig. 2, lane 5). Addition of huRPA results in crosslinking of both polβ and huRPA with the ^{32}P-labeled primer (Fig. 2, lane 6). In a control experiment performed without photoreactive NABdUTP neither polβ nor huRPA were crosslinked to the radioactive labeled primer (Fig. 2, lane 4). Furthermore, in the absence of huRPA mostly the 39 kDa polypeptide of polβ was crosslinked, while the 31 and 8 kDa proteolytic fragments of polβ were crosslinked to a lower extent (Fig. 2, lane 5). Coomassie staining of the polβ preparation revealed a small amount of these polypeptides (data not shown). In the presence of huRPA and polβ three additional polypeptides were crosslinked to the ^{32}P-labeled primer as compared with polβ alone (Fig. 2, compare lanes 5 and 6). These additional bands correspond to the p70 subunit, its proteolytic fragment and the p32 subunit of huRPA. In addition, traces of p14 subunit labeling could be detected during long gel exposures (data not shown). The intensity of crosslinking for full-length polβ and its 8 kDa fragment was decreased in the presence of huRPA (Fig. 2, compare lanes 5 and 6). Presumably the effect can be explained by competition between huRPA and DNA polymerase for the substrate.

The crosslinked products of the huRPA p32 subunit may overlap with the crosslinked products of mammalian polα (primase subunit) and 31 kDa domain of polβ. To verify whether crosslinking of the huRPA p32 subunit is a specific reaction or not we used another DNA polymerase, KF. The preparation of KF was nearly homogeneous and contained a major polypeptide of molecular mass 68 kDa as judged by gel electrophoresis (data not shown). Absence of any extra polypeptides of molecular weights within the range 30–50 kDa prompted us to analyze UV crosslinked products for KF in the absence and presence of huRPA (Fig. 2, lanes 8 and 9). The results show a distinct radiolabeled band corresponding to a product of 40 kDa, which was presumably the huRPA p32 subunit crosslinked to the radiolabeled primer. Furthermore, crosslinking of the p32 subunit was confirmed by immunoprecipitation assay (data not shown).

These results clearly demonstrate that the huRPA p70 and p32 subunits are labeled with template–primer DNA duplex carrying a photoreactive moiety at the 3′-end of the primer synthesized by the DNA polymerase *in situ*. Labeling of the p14 subunit is usually very weak in comparison with labeling of the p70 and p32 polypeptides. This labeling could be detected only after long gel exposures (data not shown). The template strand has an extension of 19 bases over the 3′-end of the primer and presumably is long enough for binding of huRPA to the single-stranded tail (42). huRPA subunits covalently crosslinked to the photoreactive primer upon UV irradiation.

Recently it was reported that huRPA interacts with several replication and transcription factors (43). Therefore, we asked the question whether the DNA polymerase–huRPA interaction can influence affinity labeling of huRPA with photoreactive DNA. To study this effect we performed UV crosslinking of polβ and huRPA under various conditions (Fig. 3). Concentrations of polβ, template–primer and huRPA used for the reaction were 5.0, 0.4 and 0.64 µM respectively. The excess polβ should move the equilibrium towards a polβ–DNA complex rather than towards the huRPA–DNA complex and therefore should shift the equilibrium towards the complex of polβ with the photoreactive DNA duplex. However, affinity of the proteins for DNA also influences competition between polβ and huRPA for DNA

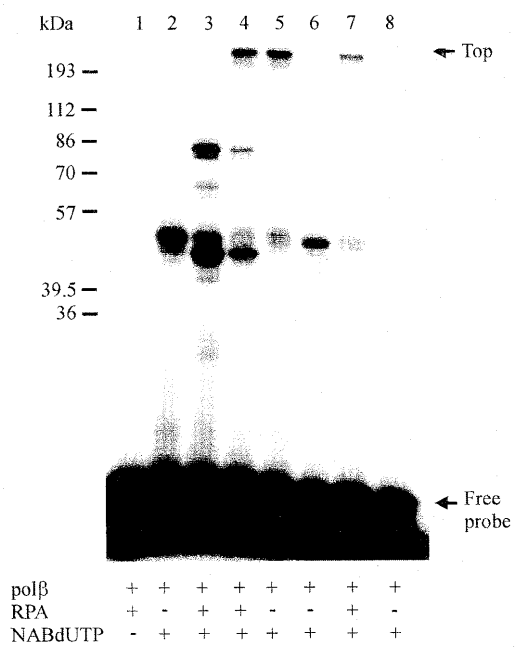

Figure 3. Photoaffinity labeling of huRPA with photoreactive primers is independent of inactivation of polβ. To analyze factors influencing affinity labeling huRPA was crosslinked to photoreactive primers in the presence of native or denatured polβ. All reaction mixtures contained 5 μM template–primer (system 1) and were incubated at 25°C for 40 min. Then they were either directly subjected to UV irradiation (lanes 1, 2, 3, 6 and 8) or heated for 2 min in boiling water, cooled and UV irradiated (lanes 4, 5 and 7). An aliquot of 0.8 μM huRPA, either native (lanes 1, 3, 4 and 8) or heat denatured (lane 7), was added to the mixture before UV crosslinking. In lanes 2–7 40 μM NABdUTP was present, but in lane 6 the photoreactive reagent (40 μM) was boiled for 2 min before incubation. In lane 8 polβ was heated for 2 min, cooled, incubated with template–primer and NABdUTP and then UV irradiated. The UV irradiation was performed with UV light, $\lambda_{315\ nm}$. The UV crosslinked products were separated by SDS–PAGE and visualized by autoradiography.

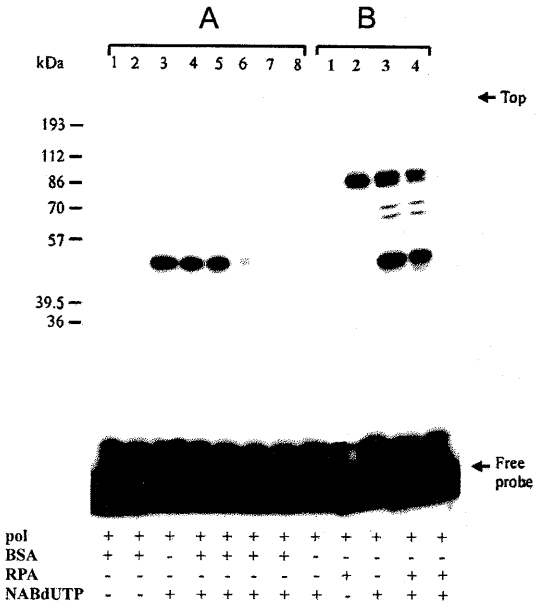

Figure 4. Photoaffinity labeling of polβ in the presence of BSA. To determine specificity of the crosslinking experiments polβ or KF was incubated with BSA and huRPA under various conditions and UV irradiated. (**A**) In lanes 1–8 the reaction mixtures contained 2.5 μM polβ and 0.56 μM template–primer (system 1). In addition, they had either 100 (lanes 1, 4 and 6) or 10 μM (lanes 2, 5 and 7) or no BSA (lanes 3 and 8). An aliquot of 40 μM NABdUTP was added to the reaction mixtures of lanes 3–8. The reaction mixtures were incubated at 25°C for 30 min before UV illumination. In lanes 6–8 polβ was heated in boiling water for 2 min without BSA and cooled, then BSA (lanes 6 and 7) or no BSA (lane 8) was added before UV illumination, which was performed as described. (**B**) In parallel, KF and huRPA were UV crosslinked and used as positive controls. Lanes 1–4, KF (1 μM) and template–primer (system 1, 0.56 μM) were incubated at 25°C for 30 min in the presence of 40 μM NABdUTP (lanes 2–4) and 0.64 μM huRPA (lane 3). Then the mixtures were UV irradiated. In lane 4 0.64 μM huRPA was added before UV illumination but after KF was heated in boiling water for 2 min and then cooled. The UV-crosslinked protein–DNA complexes were separated by SDS–PAGE and visualized by autoradiography. The position of the free probe and the molecular weight markers are indicated.

binding and complex formation of DNA with both polβ and huRPA or even a polβ–RPA interaction cannot be excluded.

When polβ was heat denatured after completing primer synthesis and then huRPA was added before subsequent irradiation of the mixture a decrease in huRPA labeling was clearly observed (Fig. 3, compare lanes 3 and 4). However, when NABdUTP was heated before incubation of the reaction mixture, followed by UV irradiation, a decrease in polβ labeling was observed (Fig. 3, compare lanes 6 and 2). Thus the explanation may be heat inactivation of photoreactive groups. In addition, heating of the reaction mixtures generated radioactive aggregates of polβ that did not penetrate into the gel, which led to a decrease in labeled band intensities (Fig. 3, lanes 4, 5 and 7). Therefore, under the experimental conditions used protein–protein interactions have very little influence, if present at all. Additional experiments are necessary to clarify the contribution of DNA polymerases to the efficiency of huRPA interaction with the template–primer DNA duplex.

Specificity of UV crosslinking of DNA to huRPA

To determine the specificity of protein–nucleic acid crosslinking we analyzed the influence of bovine serum albumin (BSA, 68 kDa) at two concentrations, 10 and 100 μM, on the crosslinking pattern. Figure 4A (lanes 3–5) shows the products of UV crosslinking of polβ with the photoreactive DNA duplex either without BSA (lane 3) or in its presence at 100 (lane 4) and 10 μM (lane 5). Figure 4 (lanes 6–8) demonstrates the results of crosslinking obtained for polβ inactivated by heating performed before the UV irradiation step in the absence of BSA (lane 8) or in its presence at 100 (lane 6) and 10 μM (lane 7); labeling of BSA could not be detected under any conditions used. To demonstrate that under similar conditions huRPA is efficiently labeled by UV irradiation experiments were carried out using KF and huRPA and run on the same gel (Fig. 4B, lanes 1–4). In the presence of 0.64 μM huRPA the large p70 subunit, its proteolytic fragment and the p32 subunit are efficiently crosslinked with the labeled primer (Fig. 4B, lanes 3 and 4).

Influence of DNA duplex structure on huRPA crosslinking

Since huRPA binds to ssDNA and dsDNA (43) and observing that huRPA is efficiently labeled using a template–primer with an overhang of 19 nt (system1), we wanted to determine whether huRPA could be labeled using a template–primer with only 1 nt overhang (system 2), blunt-ended by incorporation of NABdUMP.

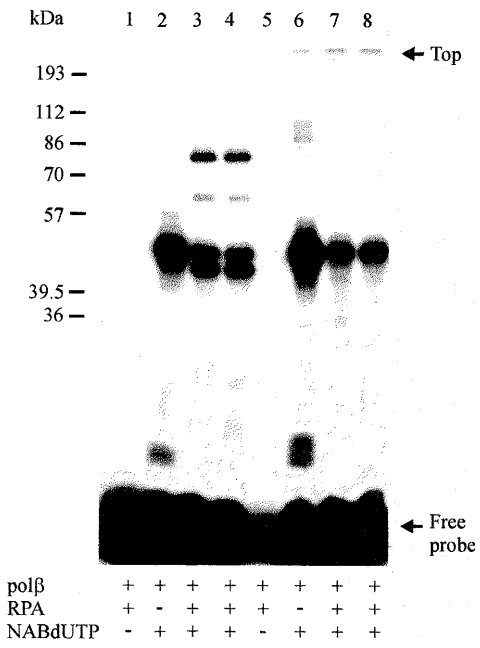

Figure 5. Human RPA is not affinity labeled by photoreactive duplex DNA without a ssDNA tail. In these sets of experiments we wanted to determine whether the affinity labeling of huRPA requires a tail of single-stranded DNA template. Polβ (5 μM) was used to elongate template–primer (system 1, 0.56 μM) (lanes 2–4) or template–primer (system 2, 0.56 μM) (lanes 6–8) with NABdUTP (40 μM) with and without RPA. huRPA (0.64 μM) was added to the reaction mixtures (lanes 1, 3–5, 7 and 8). Control reaction mixture contained polβ, huRPA and template–primer (system 1) (lane 1) or template–primer (system 2) (lane 5) without NABdUTP. In lanes 3 and 7 huRPA was present from the beginning of incubation, while in lanes 4 and 8 huRPA was added to the reaction mixtures immediately before UV irradiation. Reaction mixtures were incubated for 35 min at 25°C and then UV irradiated for 15 min at $\lambda_{315\ nm}$. The UV-crosslinked protein–DNA complexes were separated by SDS–PAGE and visualized by autoradiography. The positions of the free probe and protein markers are indicated.

Extensive polβ modification is achieved in both systems (Fig. 5, lane 2, lane 6). However, huRPA is not labeled by the blunt-ended photoreactive DNA duplex (Fig. 5, lanes 7 and 8), neither when huRPA was added together with polβ during primer elongation (lane 7) nor when it was added just prior to the UV irradiation step (lane 8). In contrast, under similar conditions using system 1 instead of system 2 huRPA is efficiently crosslinked (Fig. 5, lanes 3 and 4). These results clearly suggest that huRPA crosslinking to the photoreactive primer occurs only when the ssDNA part has a long enough extension over the duplex strand. Crosslinking of polβ serves as an internal control, since the enzyme is labeled using both systems (Fig. 5, lanes 2–4 and 6–8). It is worth noting the decrease in polβ labeling in the presence of huRPA in both template–primer systems (Fig. 5, lanes 3, 4, 7 and 8). It might be a consequence of competition between huRPA and DNA polymerase for DNA binding. The decrease in polβ and KF labeling in the presence of huRPA is also seen in the data presented in Figure 2 (compare lanes 5 and 6 and 8 and 9). At the same time, an influence of huRPA on the activity of DNA polymerase by protein–protein interactions cannot be excluded as an alternative mechanism.

DISCUSSION

There is strong biochemical and genetic evidence which shows that all three subunits of huRPA are required for its productive function (43 and references therein). It was demonstrated by various techniques that the 70 kDa subunit has intrinsic DNA binding activity (15,16,34,43–45). In particular, residues 1–441 of the p70 subunit are required for DNA binding activity, while the C-terminus of p70 is essential for stable interactions with the other two subunits of huRPA, p32 and p14 (44). However, the exact role of these two subunits remains unknown. It was shown that huRPA mutants of p32 manifest lower DNA binding activity (20), moreover ssDNA promotes p32 proteolytic digestion (44). These observations can be explained by indirect influence of the p32 subunit on complex formation between the p70 subunit and ssDNA or vice versa. Previous studies showed either an absence of binding of the p32 subunit to ssDNA (15,16,34,44,45) or a very low level (20). In the latter case affinity labeling of p32 with ssDNA is about two orders of magnitude lower than that of p70 (20).To analyze the arrangement of huRPA subunits on the DNA template and to study their specific role in interaction with DNA, photoreactive DNA duplex with a single-stranded extension was used as the main tool.

Using radioactively labeled photoreactive DNA the p32 subunit becomes highly radioactively labeled after UV irradiation (Figs 2–5). In addition, the p70 subunit and its proteolytic fragment were also labeled in our experiments, but to a lower extent than p32 (Figs 2, 4 and 5). The findings can be explained by the assumption that huRPA, presumably the p70 subunit, binds first to the ssDNA tail of the template–primer system. Subsequently, UV irradiation results in crosslinking of photoreactive primer to the p32 and p70 subunits. DNA crosslinking with huRPA is a specific reaction, since addition of BSA to the reaction mixture in 20-fold molar excess did not result in labeling with the photoreactive primer synthesized by polβ (Fig. 4). The remarkable difference in efficiency of cross-linking for different huRPA subunits also suggests that crosslinking of huRPA is a specific reaction.

In addition, we determined a decrease in the amount of crosslinking of polβ and KF in the presence of huRPA. These results suggest that the DNA polymerase and huRPA compete for interaction with the DNA substrate (Figs 3–5). When polβ was used to synthesize the photoreactive primer (Figs 2 and 5) labeling of its 8 kDa fragment was also observed, albeit to a very low extent as compared with intact polβ. The 8 kDa domain of polβ plays an essential role in internal binding to ssDNA (29). The 8 kDa polypeptide is generated in the polβ preparation by partial digestion that yields two main fragments of 31 and 8 kDa. Our crosslinking studies show that there is a clear difference in DNA crosslinking with the 8 kDa fragment in the absence or presence of huRPA (Figs 2 and 5). These findings suggest that RPA also competes with the 8 kDa domain for binding to the photoreactive DNA duplex.

This study demonstrates that a photoreactive primer synthesized *in situ* by DNA polymerases can be used for subsequent analysis of the interaction of huRPA with DNA. Our data are summarized in a model presented in Figure 6. The findings suggest a major contribution of the huRPA p70 and p32 subunits to the protein–DNA interaction. Both subunits, p70 and p32, are located in close proximity to the 3′-end of the photoreactive primer. The p14 subunit was labeled to a minor extent and we conclude that p14 is presumably located further from the 3′-end of the primer or protected by other huRPA subunits from efficient modification by the photoreactive primer, but we cannot exclude possible interaction of the 3′-end of the primer with the p14 subunit.

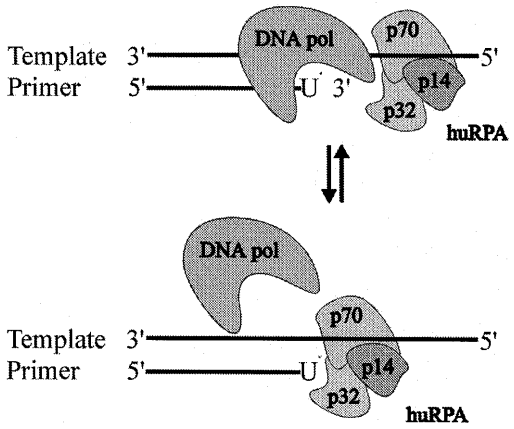

Figure 6. Model for interaction of huRPA with a DNA template–primer system. p70 binds to the template extension, while p32 localizes near the 3′-end of a primer synthesized by DNA polymerases. p14 is located further from the 3′-end of the primer. In addition, huRPA and the DNA polymerase compete for DNA binding and crosslinking at the 3′-end of the primer.

Finding a close positioning of huRPA and especially its p32 subunit to the 3′-end of the DNA duplex reveals new views into the functional activities of RPA subunits and the complex itself. Further investigations under various conditions and DNA template–primer structures need to be carried out to improve our understanding of the particular interaction of huRPA with newly synthesized DNA and its role in DNA-mediated genetic processes. Moreover, this approach can be used for analysis of DNA polymerase interactions with various proteins needed for DNA replication and/or repair *in vitro* and *in vivo*.

NOTE ADDED IN PROOF

It has recently been shown that the 32 kDa subunit of RPA monitors growing RNA–DNA primers within replicating SV-40 chromosomes (G. Kaufmann, manuscript in preparation).

ACKNOWLEDGEMENTS

This work was supported in part by a grant from MENESR (France) to O.I.Lavrik, a grant from the Russian Foundation for Basic Research (96-04-50114), a grant from RFFI-DFG (96-04-00133) and grants from the Deutsche Forschungsgemeinschaft (Gr895/11-1 and Na190/10-1). The authors are grateful to Dr W.Wlassoff for synthesis of NABdUTP, to Caroline Perrin for excellent technical assistance, to Dr Egor Vassetzkii for helping in immunoprecipitation experiments and to Drs Dmitry Cherny and Richard Smith for carefully reading the manuscript.

REFERENCES

1 Fairman,M.P. and Stillman,B. (1988) *EMBO J.*, **7**, 1211–1218.
2 Wobbe,C.R., Weissbach,L., Borowiec,J.A., Dean,F.B., Murakami,Y., Bullock,P. and Hurwitz,J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1834–1838.
3 Wold,M.S. and Kelly,T.J. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2523–2527.
4 Dean,F.B., Bullock,P., Murakami,Y., Wobbe,C.R., Weissbach,L. and Hurwitz,J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 16–20.
5 Melendy,T. and Stillman,B. (1993) *J. Biol. Chem.*, **268**, 3389–3395.
6 Tsurimoto,T., Fairman,M.P. and Stillman,B. (1989) *Mol. Cell. Biol.*, **9**, 3839–3849.
7 Wold,M.S., Li,J.J. and Kelly,T.J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 3643–3647.
8 Dornreiter,I., Erdile,L.F., Gilbert,I.U., von Winkler,D., Kelly,T.J. and Fanning,E. (1992) *EMBO J.*, **11**, 769–776.
9 Erdile,L.F., Heyer,W.D., Kolodner,R. and Kelly,T.J. (1991) *J. Biol. Chem.*, **266**, 12090–12098.
10 Kenny,M.K., Lee,S.-H. and Hurwitz,J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9757–9761.
11 Lee,S.-H., Pan,Z.Q., Kwong,A.D., Burgers,P.M.J. and Hurwitz,J. (1991) *J. Biol. Chem.*, **266**, 22707–22717.
12 Tsurimoto,T. and Stillman,B. (1989) *EMBO J.*, **8**, 3883–3889.
13 Brill,S.J. and Stillman,B. (1989) *Nature*, **342**, 92–95.
14 Brill,S.J. and Stillman,B. (1991) *Genes Dev.*, **5**, 1589–1600.
15 Wold,M.S., Weinberg,D.H., Virshup,D.M., Li,J.J. and Kelly,T.J. (1989) *J. Biol. Chem.*, **264**, 2801–2809.
16 Kenny,M.K., Schlegel,U., Furneaux,H. and Hurwitz,J. (1990) *J. Biol. Chem.*, **265**, 7693–7700.
17 Din,S.-U., Brill,S.J. and Stillman,B. (1990) *Genes Dev.*, **4**, 968–977.
18 Dutta,A., Din,S.-U., Brill,S.J. and Stillman,B. (1991) *Cold Spring Harbor Symp. Quant. Biol.*, **56**, 315–324.
19 Dutta,A. and Stillman,B. (1992) *EMBO J.*, **11**, 2189–2199.
20 Philipova,D., Mullen,J., Maniar,H., Lu,J., Gu,C. and Brill,S. (1996) *Genes Dev.*, **10**, 2222–2233.
21 Stigger,E., Dean,F., Hurwitz,J. and Lee,S.-H. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 579–583.
22 Henricksen,L.A., Umbricht,C.B. and Wold,M.S. (1994) *J. Biol. Chem.*, **269**, 11121–11132.
23 Wlassoff,W.A., Dobrikov,M.I., Safronov,I.V., Dudko,R.Y., Bogachev,V.S., Kandaurova,V.V., Shishkin,G.V., Dymshits,G.M. and Lavrik,O.I. (1995) *Bioconjugate Chem.*, **6**, 352–360.
24 Doronin,S.V., Dobrikov,M.I. and Lavrik,O.I. (1992) *FEBS Lett.*, **313**, 31–32.
25 Lavrik,O.I., Prasad,R., Beard,W.A. Safronov,I.V., Dobrikov,M.I., Srivastava,D.K., Shishkin,G.V., Wood,T.G. and Wilson,S.H. (1996) *J. Biol. Chem.*, **271**, 21891–21897.
26 Doronin,S.V., Dobrikov,M.I., Buckle,M., Roux,P., Buc,H. and Lavrik,O.I. (1994) *FEBS Lett.*, **354**, 200–202.
27 Scherbik,N.V., Khodyreva,S.N., Wlassoff,W.A., Dobrikov,M.I., Dymshits,G.M. and Lavrik,O.I. (1997) *Mol. Biol.* (*Moscow)*, **31**, 344–352.
28 Stadlbauer,F., Brueckner,A., Rehfuess,Ch., Eckerskorn,Ch., Lottspeich,F., Förster,V., Tseng,B.Y. and Nasheuer,H.-P. (1994) *Eur. J. Biochem.*, **222**, 781–793.
29 Brueckner,A., Stadlbauer,F., Guarino,L.A., Brunahl,A., Schneider,C., Rehfuess,C., Prives,C., Fanning,E. and Nasheuer,H.-P. (1995) *Mol. Cell. Biol.* **15**, 1716–1724.
30 Stadlbauer,F., Voitenleitner,C.F., Brueckner,A., Fanning,E. and Nasheuer,H.-P. (1996) *Mol. Cell. Biol.*, **16**, 94–104.
31 Beard,W.A. and Wilson,S.H. (1995) *Methods Enzymol.*, **262**, 98–107.
32 Joyce,K.M. and Grindley,D.F. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1830–1834.
33 Henricksen,L.A., Umbricht,C.B. and Wold,M.S. (1994) *J. Biol. Chem.*, **269**, 24203–24208.
34 Nasheuer,H.-P., von Winkler,D., Schneider,C., Dornreiter,I., Gilbert,I. and Fanning,E. (1992) *Chromosoma*, **102**, S52–S59
35 Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds) (1989) *Current Protocols in Molecular Biology.* John Wiley & Sons, New York, NY.
36 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
37 Beard,W.A. and Wilson,S.H. (1993) *Biochemistry*, **32**, 9745–9753.
38 Georgaki,A., Strack,B., Podust,V. and Hübscher,U. (1992) *FEBS Lett.*, **308**, 240–244.
39 Treuner,K., Ramsperger,U. and Knippers,R. (1996) *J. Mol. Biol.*, **259**, 104–112.
40 Prasad,R., Kumar,A., Widen,S.G., Casas-Finet,J.R. and Wilson,S.H. (1993) *J. Biol. Chem.*, **268**, 22746–22755
41 Liu,D., Prasad,R., Wilson,S.H., DeRose,E.F. and Mullen,G.P. (1996) *Biochemistry*, **35**, 6188–6200.
42 Blackwell,L.J., Borowiec,J.A. and Mastrangelo,I.A. (1996) *Mol. Cell. Biol.*, **16**, 4798–4807.
43 Wold,M.S. (1997) *Annu. Rev. Biochem.*, **66**, 61–92.
44 Gomes,X.V., Henricksen,L.A. and Wold,M.S. (1996) *Biochemistry*, **35**, 5586–5595.
45 Gomes,X.V. and Wold,M.S. (1995) *J. Biol. Chem.*, **270**, 4534–4543.
46 Kim,Ch., Paulus,B.F. and Wold,M.S. (1994) *Biochemistry*, **33**, 14197–14206.