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REVIEW

Eucaryotic DNA Replication Complex: Study of Structure and Function Using the Affinity Modification Technique

D. Yu. Khlimankov, N. I. Rechkunova, and O. I. Lavrik*

Novosibirsk Institute of Bioorganic Chemistry, Siberian Division, Russian Academy of Sciences, pr. Lavrentieva 8, Novosibirsk 630090, Russia; fax: (3832) 333-677; E-mail: lavrik@niboch.nsc.ru

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Abstract—Eucaryotic DNA replication complex is now one of the most intensively studied subjects of molecular biology and biochemistry. In addition to detailed studies on the structures and functions of individual DNA polymerases involved in this process, other enzymes and protein factors are also given much attention. The structures and functions of proteins in the replication complexes are studied by various approaches, including X-ray diffraction analysis. At present, this approach provides sufficient information about the structures and functions of individual biopolymers and their complexes with ligands. However, this approach is unsuitable for studies on proteins, which cannot be cloned and isolated in amounts sufficient for X-ray diffraction analysis. Moreover, this approach is inapplicable for studies on multicomponent systems, such as DNA replication and repair complexes. Furthermore, data of X-ray diffraction analysis virtually never characterize the variety of dynamic interactions in enzymatic systems. Affinity modification is an alternative and rather successful approach for studies on structure-functional organization of supramolecular structures. This approach can be used for studies on individual enzymes and their complexes with substrates and also on systems consisting of numerous interacting proteins and nucleic acids. The purpose of this review is to analyze the available data obtained by affinity modification studies on the eucaryotic replication complex.

Key words: DNA replication, DNA polymerases, replication factors, affinity modification

The genetic stability of living organisms significantly depends on the functions of the protein complex responsible for DNA replication. Genomic DNA is synthesized by DNA polymerases with involvement of proteins providing for the interaction of DNA polymerases with DNA and processivity of the synthesis. The concurrent synthesis of both DNA chains in higher eucaryotes is described by a model of the replication fork (Fig. 1) [1, 2]. According to this model, the double helix of DNA is unwound during the initiation stage under the action of DNA-helicase and replication protein A (RPA). Then DNA polymerase α-primase (Pol α) synthesizes RNA primers, which in their turn are primers for synthesis of RNA–DNA primers. Thus, DNA polymerase α in the complex with the unwound DNA realizes two functions: the synthesis of RNA–DNA primers on the DNA leading strand and the concurrent synthesis of short RNA–DNA primers (precursors of Okazaki fragments) on the lagging strand. On the replication system of virus SV40 the lagging strand was shown to be primed rather often at the distance of ~50 nucleotides (nt) between the initiation sites. Precursors of Okazaki fragments consist of the RNA primer of ~10 nt elongated by 10-20 deoxyribonucleotides. Pol α functions in the absence of the proliferating cell nuclear antigen (PCNA) and replication factor C (RFC). On the contrary, DNA polymerase δ (Pol δ) depends on PCNA and RFC—factor, which promotes the PCNA boarding onto DNA.

Because precursors of Okazaki fragments are produced in the absence of ATP, and RFC is known to need ATP for interaction with DNA polymerases, the precursors are produced only under the action of DNA polymerase α-primase independently of RFC. After the boarding onto DNA, Pol δ and PCNA provide for the inclusion of an additional 10-20 nt into the primer. As a result, the 3′-end of the growing primers achieves the RNA primer of the next precursor of an Okazaki fragment. Then the primer is elongated farther during or after the removal of the RNA primer, and nick-transla-

Abbreviations: RPA) replication protein A; RFC) replication factor C; Pol α) DNA polymerase α-primase; Pol δ) DNA polymerase δ; Pol ε) DNA polymerase ε; FEN-1) flap endonuclease 1; PCNA) proliferating cell nuclear antigen; DBD) DNA-binding domain; APE-1) apurinic/apyrimidinic endonuclease-1.

* To whom correspondence should be addressed.

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tional synthesis seems to occur until the first deoxyribonucleotide of the next primer. According to the accepted model, RNase H1 removes the RNA primer from the RNA–DNA primer and retains one ribonucleotide link which is later removed by flap endonuclease 1 (FEN-1). DNA 2 helicase/endonuclease was recently shown to interact with FEN-1 and replace RNase H1, which suggests another model for processing of Okazaki fragments. According to this model, a successive action of FEN-1 and DNA 2 helicase/endonuclease is necessary to remove the RNA primer [3]. Okazaki fragments are supposed to mature under conditions of coordinated action of DNA polymerases, PCNA, RFC, RPA, RNase H1, FEN-1, DNA 2 helicase/endonuclease, and DNA ligase I [3, 4]. Twenty-three proteins were recently found to be involved in the DNA synthesis on the lagging strand [5]. DNA ligase I is one of the components required for joining of Okazaki fragments. This enzyme also interacts with PCNA and influences the PCNA-dependent DNA synthesis with involvement of Pol δ and Pol ε [6, 7]. According to one hypothesis, PCNA functions in the replication fork as a binding link between DNA polymerases and other factors responsible for coordination of elongation and processing of the lagging strand [8]. According to another hypothesis, the replication protein A is the most important factor which coordinates actions of all other proteins in the switching of the DNA synthesis from one DNA polymerase to another and termination of the synthesis [4, 9, 10].

Obviously, DNA replication is regulated by numerous protein—protein and DNA—protein interactions, but their mechanisms remain unknown. New eucaryotic DNA polymerases have been recently discovered, but their role in the replication complex has not been determined [2, 11]. Therefore, a careful review of protein ensembles responsible for replication seems reasonable. Moreover, the DNA replication complex functions in coordination with protein ensembles, which provide for repair of DNA damage. Consequently, the supramolecular machine metabolizing the genomic DNA and providing for stable reproduction of its structure appears to be still more complicated. The eucaryotic replication complex is unlikely to be an ordered structure. More likely, functional ensembles of proteins are produced during their interaction with DNA, and their composition depends on transformations of DNA.

The replication complex is complicated and dynamic, therefore, its study by X-ray diffraction method and other instrumental techniques meets some limitations, and the development and use of alternative approaches are needed. Affinity modification is a method successfully used for studies on structure and functions of replication and repair complexes and also of ribosomes [12-17]. Advantages of affinity modification and difficulties associated with interpretation of the findings are analyzed in detail in some reviews [15-17]; thus, in the present review we shall pay less attention to these aspects.

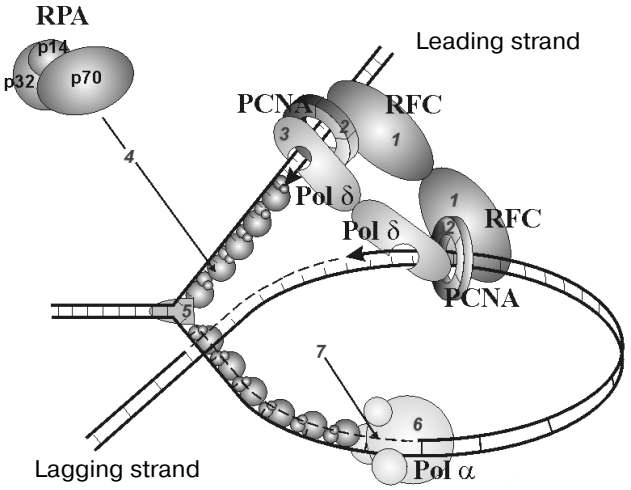

Fig. 1. Hypothetic model of eucaryotic replication fork which includes several DNA polymerases [1]: RFC (*1*), PCNA (*2*), Pol δ(ε) (*3*), RPA (*4*), helicase (*5*), DNA polymerase α-primase (*6*), the RNA–DNA primer (*7*).

The present review covers the results of studies on the replication complex proteins, which have been performed using the method of affinity modification within the last 20 years.

DNA POLYMERASES AND THE REPLICATIVE FORK FACTORS

DNA polymerase α-primase. DNA polymerase α was the first DNA polymerase detected in eucaryotic cells [18, 19]. This enzyme is mainly responsible for the polymerase activity in extracts from proliferating cells under standard conditions of testing on the activated DNA. The protein consists of four subunits: a large catalytic subunit (165-182 kD), a subunit with the lower molecular weight of 68-70 kD, and two small subunits of ~48-50 and 55-60 kD. The DNA polymerase activity is inherent in the large subunit. The subunit with molecular weight of 68-70 kD is thought to be necessary to transfer the catalytic polypeptide into the cell nucleus. It is also involved in the regulation of the Pol α level in the cell, because this subunit stimulates the synthesis of the catalytic polypeptide. The two small subunits have the primase activity. And the 48-kD subunit has catalytic properties and provides for the priming of DNA, whereas the 58-kD subunit is involved in binding the initiating purine nucleotide and interaction of the 48-kD subunit with the catalytic subunit of the enzyme. Pol α produces functional complexes with a number of enzymes and auxiliary proteins. These complexes seem to be fragments of the replication ensem-

ble of the cells. It is supposed that Pol α interacts with Pol δ in the complex responsible for replication of the leading strand and with Pol δ or Pol ε in the complex synthesizing the lagging strand [18].

Affinity modification of DNA polymerase α-primase. Interactions of Pol α with templates, primers, and deoxynucleoside-5′-triphosphates were studied by an approach based on the competition of substrates of DNA polymerases with affinity reagents specifically interacting with DNA- or dNTP-binding sites of DNA polymerases [20]. This approach was formulated in general terms in [15]. Specific features of the template binding were studied using an affinity reagent based on a decanucleotide containing the reactive residue of *cis*-aquadihydroxydiaminoplatinum. Its interaction with the enzyme was followed by inactivation of the DNA polymerase activity. A variety of oligonucleotides of different length and composition were studied as competitive inhibitors, and thus effects of the length and structure of oligonucleotides on their complexing with the DNA-binding site of Pol α were determined [21-23]. Based on the findings, a hypothetical model of the template-primer interaction with Pol α was proposed (Fig. 2). According to this model, both the template and primer forms with the enzyme a Me^{2+}-dependent electrostatic contact and a hydrogen bond produced by the 3′-terminal nucleotide of the primer and one of the internucleotide phosphates of the template. Nucleotide components of the template seem to interact with the enzyme by hydrophobic binding. These weak interactions are summarized with elongation of the DNA chain that results in its efficient interaction with the active site of DNA polymerase. Other monomeric links of the primer do not interact with the enzyme, but produce Watson—Crick hydrogen bonds. The decisive role of interaction of the template and primer with the enzyme in formation of the dNTP-binding site of DNA polymerases was also shown. The dNTP γ-phosphate was shown to significantly contribute to the template-dependent specific adjustment of the template-complementary dNTP [20]. The significant contribution of the

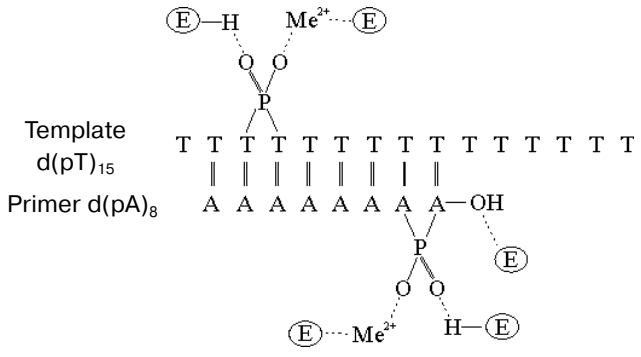

Fig. 2. The hypothetical scheme of template-primer interaction with DNA polymerase α [20-22].

3′-terminal end of the primer to the efficiency of its interaction with DNA-polymerases was confirmed by other approaches [24].

Enzymes are highly selectively and efficiently modified by an approach based on their ability to change substrates, which can be specifically bound. Affinity reagents used for this labeling have no reactive groups, but covalently bind to the enzyme due to the catalytic change realized by the enzyme [15]. Such reagents are called suicidal. The absence of active groups in the initial affinity reagent prevents side transformations of the reagent and the nonspecific labeling of the enzyme is virtually excluded. Such technique of affinity labeling was used for DNA polymerases α and β. The so-called suicidal reagent adenosine-2′,3′-riboepoxide-5′-triphosphate (epATP) inactivated human Pol α and Pol β and DNA polymerase of avian myeloblastosis virus [25]. It was suggested that epATP should be included into the 3′-end of the primer during the first stage. Then, after translocation of the template-primer complex, the enzyme should be alkylated by the epoxy group of the primer. Pol α from human placenta was studied using epATP [26, 27]. The enzyme was inactivated only in the presence of the complementary template, primer, and Mg^{2+}. Assuming the suicidal mechanism of the affinity modification, the possibility of selective radioactive labeling of the protein was studied using 5′-[^{32}P]d$(pA)_{10}$ in the presence of poly(dT)-template and epATP. EpATP was shown to be an affinity reagent dependent on the template and primer, and not a suicidal substrate as thought earlier.

Studies on the dNTP-binding sites of DNA polymerase α-primase from *Drosophila melanogaster* have been performed using pyridoxal-5′-phosphate (PLP) [28, 29], a known natural cofactor efficiently inhibiting various nucleotide-binding enzymes via production of Schiff bases with ε-amino groups of lysine residues. PLP is not an affinity reagent in full measure because it has no nucleotide residue in its structure. More likely, it is a group-specific reagent for lysine residues. Nevertheless, this reagent can be used to modify nucleotide-recognizing sites of enzymes. PLP reversibly inhibits both DNA-polymerase and primase activities of the enzyme due to interactions with two sites. One of them, the site PLP1, is located on the subunit with molecular weight of 46 kD and seems to relate to the primase site. In the presence of any dNTP, this site is protected against modification, and inactivation of the primase activity is also prevented. The second dNTP-binding site, PLP2, is located on the large subunit (148 kD). This site is coupled with the DNA polymerase site of the enzyme: the modification and inactivation can be prevented only in the concurrent presence of the template-primer complex and complementary dNTP.

Photoaffinity labeling was used to study the dNTP-binding site of Pol α, with an analog of dTTP, 1-β-arabinofuranosyl-5-(E)-(4-azidostyryl)uracyl-5′-triphosphate

(aradUTP), as a reagent [30]. Irradiation of the enzyme in the presence of this analog inhibited the polymerase activity. Moreover, natural dTTP competed in the dark with aradUTP for binding to DNA polymerase. Using the radioactively labeled [γ^{32}-P]aradUTP, the authors found that the affinity labeling of the enzyme occurred only in the presence of the primer-template (poly-dA–oligo-dT); in the presence only of DNA-template efficiency of the labeling was significantly lower. Therefore, it was suggested that dNTP should be efficiently bound in the active site only in the case of the enzyme binding to the template, but additional conformational changes and a subsequent formation of the dNTP-binding site could occur only in the presence of the template-primer complex. Thus, findings of [30] confirmed the data of [20, 21] on the decisive role of the interaction between the template-primer complex and polymerases in formation of the dNTP-binding site of DNA polymerases.

As mentioned, eucaryotic DNA polymerase α-primase consists of the polymerase subunit with molecular weight of 120-180 kD, the subunit of 72 kD with unknown function, and two primase subunits with molecular weights of 59 and 49 kD. For affinity modification of Pol α from human placenta exo-*N*-[2-(*p*-azidotetrafluorobenzamido)-ethyl)]deoxycytidine-5′-triphosphate (FABdCTP) was used [31], which was covalently bound to the protein and then incorporated into the 3′-end of the primer by the activity of DNA polymerase α that modified the 165- and 49-kD subunits. On the other hand, an attachment of the photoreactive primer presynthesized *in situ* in the presence of FABdCTP resulted in labeling of both primase subunits in addition to the attachment to the 165-kD subunit, but the 72-kD polypeptide remained unlabeled. Therefore, it was suggested that three subunits of the enzyme (the polymerase and two primase subunits) should be located near the 3′-end of the primer, whereas the 72-kD subunit should be far from it. This example shows that photoreactive DNA can be *in vitro* synthesized in the presence of dNTP analogs containing arylazido groups and used for modification of multisubunit enzymes. This approach was widely used later. Based on the findings, it was suggested that in the complex DNA polymerase α-primase the DNA- and RNA-polymerase sites of the enzyme should compete for the 3′-end of the primer.

Affinity modification of primase. Highly selective affinity labeling previously used for studies on functional topography of the active site of RNA polymerases was also used for studies on the active site of DNA primase from yeast [32]. Benzaldehyde derivatives of ATP, ADP, and AMP covalently bound to the active site of the primase were used as initiating substrates for synthesis of the primer in the presence of [α-^{32}P]ATP as an elongating substrate. The protein was modified with benzaldehyde derivative of adenine and then labeled by addition of [α^{32}-P]ATP and poly-dT template. The affinity labeling of both the full complex DNA polymerase α-primase and individual primase domain has shown that both primase subunits (p58 and p48) are involved in formation of the enzyme catalytic site, although a direct photoattachment of [α-^{32}P]ATP resulted in location of the ribonucleoside triphosphate-binding site only on p48 polypeptide. Based on previous data on the structure and functions of subunits of DNA polymerase α-primase [33] and their own findings, a model of functioning of eucaryotic DNA polymerase α-primase was proposed (Fig. 3) [32]. According to this model, the ribonucleotide-binding site is located on p48 polypeptide and the binding sites of the primer-

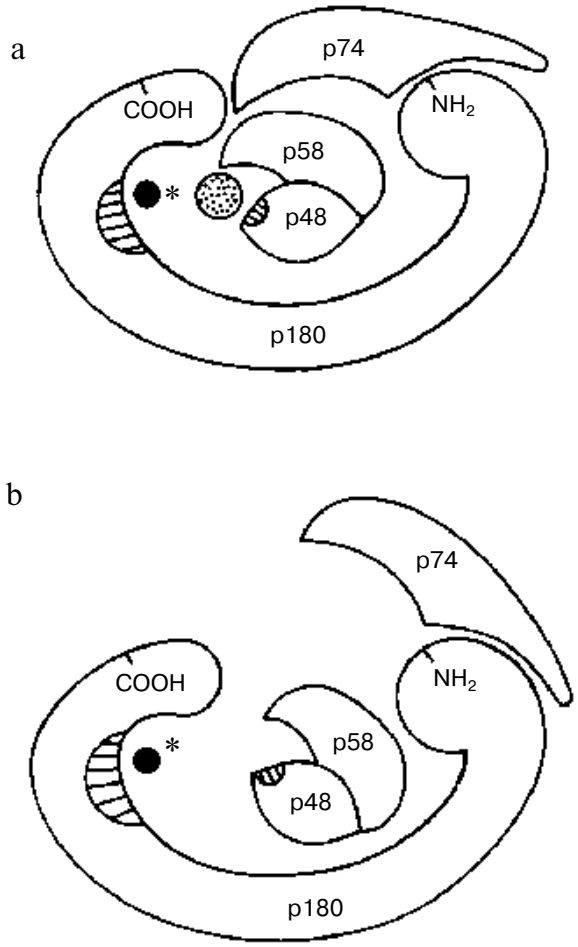

Fig. 3. Model of regulation of DNA polymerase—DNA primase activities [32]: a) initiation of replication on the DNA template; b) elongation of RNA primers. Hatched semicircles represent the DNA- and dNTP-binding sites on p180 polypeptide and the rNTP-binding site on p48 polypeptide of DNA polymerase α-primase. The light circle between p48 and p58 (model (a)) corresponds to the active site responsible for synthesis of the RNA primer. DNA template is shown by the dark circle, and the RNA primer and newly synthesized DNA are shown by the asterisk.

template complex and dNTP are located on the C terminus of p180 subunit. These sites seem to contact with the site produced by p58 and p48 subunits of DNA primase and be responsible for synthesis of the primer. DNA polymerase interacts with DNA primase with involvement of the N-terminal domain of the polymerase subunit. p74 polypeptide seems to be involved in stabilization of the polymerase—primase complex. In the presence of ribonucleoside triphosphates p74 and p180 subunits are brought into proximity, i.e., are "waiting" for termination of synthesis of RNA primers by primase. After RNA primers of a certain length have been synthesized, the 3′-end of the primer is transferred from the active site of primase onto the active site of polymerase, and this promotes this polypeptide to start the elongation of RNA primers in the presence of deoxyribonucleotides. The transfer of RNA primer and elongation in the course of replication are results of the further dissociation of the complex of the p74 and p180 subunits.

The primase domain of DNA polymerase α-primase from human placenta was also successfully studied by highly selective affinity labeling [34]. *o*-Formylphenyl-ATP, -ADP, and -AMP esters bound to the active site of primase were used as initiating substrates for synthesis of the primer in the presence of ATP as an elongating substrate. If the covalent binding to the primase active site occurred in the presence of poly-dT template, only p48 subunit was modified by all reagents. If the template was added after the covalent binding of the reagent, both subunits (p58 and p48) were modified. *o*-Formylphenyl-ADP ester modified both subunits, whereas AMP ester modified only p48. In the absence of the template, *o*-formylphenyl-ATP ester also modified both subunits. Based on these findings, it was suggested that the binding site of the initiating substrate should be located on p48 subunit in the region of its contact with p58, and this is consistent with earlier data for DNA polymerase α from yeast [32].

Interaction of the RNA primer newly synthesized by primase with the catalytic subunit of DNA polymerase is not necessary for the further regulation of the primase activity [35, 36]. However, on what subunit of the primase complex the domain regulating the further elongation of the RNA primer was located remained unclear. In [37] a DNA template was used which contained either the modified link as azidophenacyl bromide attached to the phosphothioate group, or the modified base as 5-iodo-2′-deoxyuridine or 5-bromo-2′-deoxyuridine. The photoattachment of the p58—p48 primase complex with such DNAs indicated that both individual subunits of primase interacted with single-stranded DNA, whereas in the case of the p58—p48 primase complex the cross-linking occurred only with p58 subunit. After synthesis of the primer by the primase complex, only p58 was covalently bound to DNA. Based on these findings, a model of regulation of the primer synthesis was proposed that presented the newly synthesized primer to remain bound to p58, which regulates the further synthesis of DNA. The ability of p58 to interact with the primer-template structures suggests that p58 acts as a messenger in the primer transfer from the active site of primase into the active site of DNA polymerase. Thus, various techniques of affinity labeling were used to locate the active sites on the subunits of DNA polymerase α-primase and study their interaction during the synthesis of RNA primers and their further elongation.

DNA Polymerase δ. DNA polymerase δ (Pol δ) was discovered much later than DNA polymerase α [2] because the activity of Pol δ depends on auxiliary proteins PCNA, RFC, and RPA, which are constituents of the holoenzymes (functional complexes) of this DNA polymerase. In the absence of these factors, the activity of DNA polymerase δ was virtually not detected. The holoenzyme of DNA polymerase δ is suggested to be involved in synthesis of the leading strand, whereas the holoenzyme of DNA polymerase δ or ε seems to be involved in synthesis of the lagging strand of the replication fork [18]. DNA polymerase δ is a heterotetramer consisting of a catalytic subunit with molecular weight of 125-130 kD and a subunit of 48-55 kD that is required for binding the processivity factor PCNA and also recently discovered 66- and 12-kD subunits [38, 39]. X-Ray diffraction analysis of DNA polymerase δ is associated with difficulties in its cloning and isolation, but this is also a problem in the case of DNA polymerase α-primase described earlier. Only recently all subunits of Pol δ were cloned in a baculovirus system [40]. Therefore, studies by chemical methods on this enzyme and its complexes with other replication factors are undeniably interesting.

Until recently, the relative locations of DNA polymerase δ and PCNA on DNA were unknown, as well as the role of the polymerase subunits in synthesis of DNA. *A priori*, two models of the Pol δ and PCNA location on the primer-template structure were supposed. According to the first model (Fig. 4a), PCNA is located behind polymerase, similarly to location of the β-subunit of DNA polymerase III from *Escherichia coli* [41]. The β-subunit of this enzyme performs the same function as PCNA. The second model presents PCNA located ahead of Pol δ (Fig. 4b). X-Ray diffraction studies on PCNA have shown it to be a homotrimer of 36-kD subunits, which encircles DNA as a ring-shaped structure sliding over it during the synthesis [8]. In other works reviewed in [8], Pol δ is shown to bind to the C-terminal region of PCNA. The relative location of the proteins on the primer-template was studied with 3′-biotinylated oligonucleotides as the template [42]. If the template was annealed with the short primer, addition of streptavidin resulted in a very low processivity of the polymerase that corresponded to the background processivity without the auxiliary factor PCNA. On the primer elongation to 19 nt the PCNA-dependent synthesis of Pol δ occurred even after the addi-

tion of streptavidin. Obviously, the biotin—streptavidin complex on the long primer did not prevent the PCNA association around the primed DNA, and this supported the PCNA location behind polymerase δ during the processive synthesis of DNA.

The problem of organization of the Pol δ subunits around DNA was solved using affinity modification [42]. Affinity modification of Pol δ was performed with primer-template structures containing a photoreactive group in a certain position of the template or primer. 5-(*N*-(*p*-Azidobenzoyl)-3-aminoallyl)dUMP was used as the photoreactive link. The enzyme was UV-irradiated either in the presence of single-stranded DNA containing a photoactive analog and [α-^{32}P]dCMP in the adjacent position, or in the presence of duplex of radioactively labeled photoactive DNA. The crosslinking occurred only with the large subunit of DNA polymerase δ (125 kD) that suggested a contact of this subunit with both single- and double-stranded DNA. Moreover, with antibodies specific to C and N terminuses of DNA polymerase δ the active site was detected in the C-terminal region of the molecule. Thus, just this subunit is responsible for synthesis of the primer and interaction of Pol δ with DNA, whereas the p50 subunit seems to be required for the interaction of Pol δ with PCNA (Fig. 4c) [42]. The validity of this model has to be either supported or rejected by further studies. Obviously, the discovery of new subunits in the complex of DNA polymerase δ can correct the description of its interaction with DNA template and PCNA.

Replication protein A. Replication protein A (RPA) is a key factor in eucaryotic replication. This protein consists of polypeptides with molecular weight of 70 (p70), 32 (p32), and 14 kD (p14), i.e., is a heterotrimer [10]. RPA is known to be a factor binding single-stranded DNA. The dissociation constant of RPA complexes with single-stranded DNA is 10^{-9}-10^{-11} M, whereas the affinity of RPA for double-stranded DNA is three orders of magnitude lower. At certain stages of DNA replication, recombination, or repair the complementary chains separate, and RPA stabilizes the produced single-stranded DNA regions [10].

DNA-binding domains of replication protein A from yeast cells were studied by direct photoattachment [43]. On the whole, three subunits of RPA contain four domains with primary structure similar to that of domains of the procaryotic SSB-protein from *E. coli*. These domains were considered to be DNA-binding domains (DBD). p70 subunit contains two such regions (A and B), and together they mainly contribute to the interaction with single-stranded DNA. Each of the p32 and p14 subunits contains one DBD (C and D, respectively); therefore, they bind single-stranded DNA with lower stability than in the case of p70. Later the DBD-C and DBD-D were ascribed to p70 and p32, respectively. By photoattachment of the recombinant RPA with single-stranded

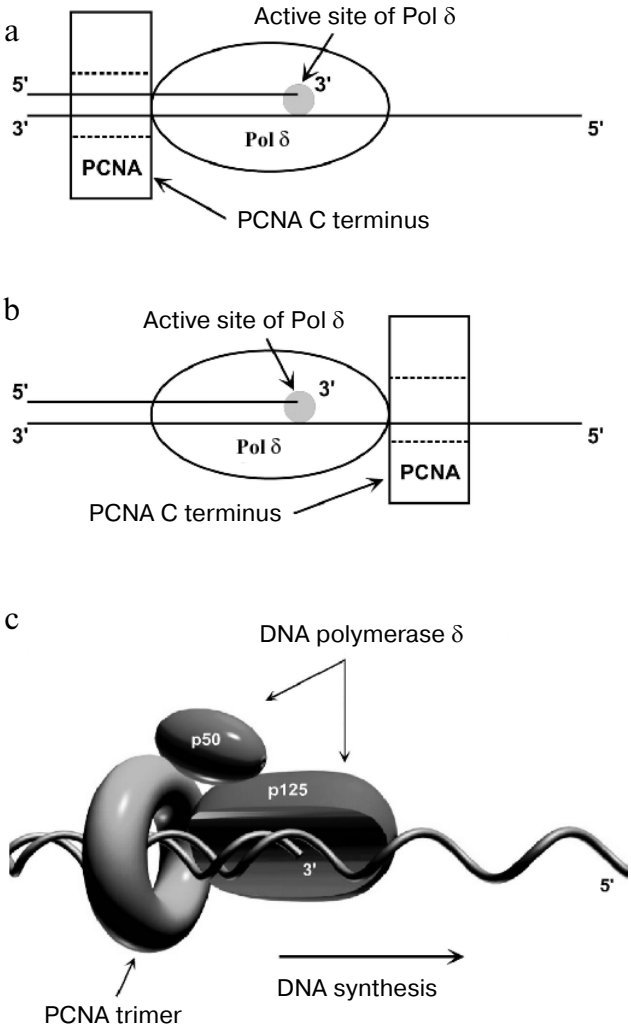

Fig. 4. Model of interaction of Pol δ and PCNA with the primer-template complex [42]. Possible orientations of Pol δ and PCNA in the complex with the primer and template are shown: PCNA behind (a) and ahead (b) of Pol δ. c) Model of the complex Pol δ—PCNA—DNA in the course of replication.

DNA each DNA-binding domain was shown to consist of ~120 amino acids which form a conservative sequence, with two aromatic amino acids in the center. Mutation of these aromatic amino acids is lethal for three domains and abolishes the DNA-binding function of the protein.

In spite of intensive X-ray diffraction analysis of human RPA and its complexes with DNA [44], the structure of the full RPA heterotrimer and its complexes with DNA is far from being finally determined. Therefore, RPA and its interaction with DNA are mainly studied by other methods [10, 45]. Photoaffinity modification with reactive intermediates of DNA replication seems to be sufficiently timely and promising for elucidation of the role of RPA subunits in its binding to DNA [46]. Even before using this approach, the main DNA-binding domains of RPA (A, B, and C) were known to be con-

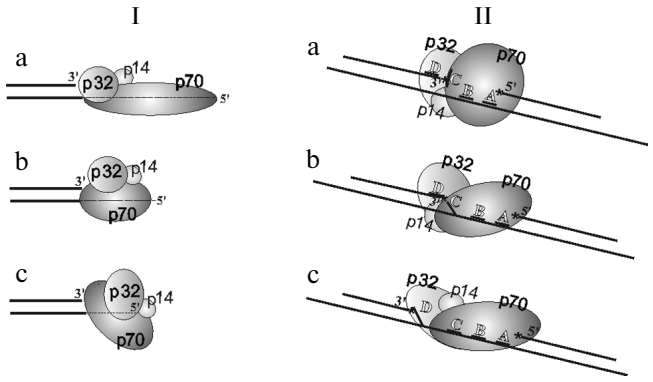

Fig. 5. I) Hypothetical model of RPA interaction with DNA duplexes containing template protruding 5′-ends of different length [47]: a) the elongated conformation of RPA (interaction with a single-stranded region of the template of 30 nt in length); b) the compact conformation of RPA (interaction with a single-stranded region of the template of 13 nt in length); c) the globular conformation of RPA (interaction with a single-stranded region of the template of 9 nt in length). II) Hypothetical model of RPA interaction with gaps of different length [49]: a) binding of gaps of 7 and 9 nt in length; b) binding of the gap of 13 nt in length; c) binding of the gap of 30 nt in length. A, B, C, and D are DNA-binding domains of RPA.

stituents of p70 [10]. Conformational rearrangements in RPA were also shown during its binding to single-stranded DNA [10]. However, contributions of p32 and p14 subunits and of DNA-binding domains of p70 to the binding to DNA, as well as their role in the RPA rearrangements associated with this binding, remained unclear. Interaction of the RPA subunits with DNA structures generated during the replication was studied using 5-[*N*-(2-nitro-5-azidobenzoyl)-*trans*-3-aminopropenyl-1)deoxyuridine-5′-monophosphate attached to the 3′-end of the radioactively labeled primer. The RPA subunits covalently bound to photoactive DNA were identified by immunoprecipitation with specific antibodies, and the RPA subunit with the middle molecular weight of 32 kD was shown to be the main product of the photoattachment [46]. These results were the first to directly indicate the role of this subunit in RPA binding to DNA and indirectly suggest the polarity of the RPA binding to the single-stranded DNA template. Furthermore, the interaction of RPA with DNA intermediates arising in the replication fork at different stages of the synthesis was modeled using DNA duplexes with protruding region of the template from 31 to 4 nt in length. In the case of photoreactive DNA duplexes with the protruding region of the template of 30 and 18 nt the covalent binding of p32 subunit to the 3′-end of the primer was mainly observed, whereas the shortening of the template strand to 13-12 nt and further mainly provided for the covalent attachment of p70. These data suggest conformational rearrangements in RPA modulated by the length of the protruding region of the template strand. By electron microscopy the RPA complex with single-stranded DNA was shown to take an elongated, contracted, or globular conformation depending on the length of the DNA [10]. In these conformations, the position of the RPA subunits relatively to the 3′-end of the growing strand was suggested to vary (Fig. 5, I). In the elongated conformation of RPA p32 is located near the 3′-end of the growing DNA strand (Fig. 5, Ia), whereas the acquisition by RPA of the contracted and then globular conformation results in drifting p32 apart from the 3′-end of DNA (Fig. 5, I, b and c). The change in the RPA conformation strictly depends on the length of the protruding region of the template, similarly for observations for dUTP analogs with the photoreactive group attached by linkers of various lengths and also on using thiothymidine-5′-triphosphate with another group in the nucleotide structure [47, 48].

The replication intermediates generated on the lagging strand of DNA were modeled by DNA structures containing gaps from 30 to 9 nt in size. It was interesting to determine the location of the RPA subunits near the 5′-end of the gap; therefore, the photoreactive residues were introduced by synthesis into the 5′-end of the primer framing the gap at the 5′-end.

With these DNA structures, the efficiency of p32 labeling was higher for the elongated gap and decreased with decrease in the gap size if the photoactive nucleotide was located on the 3′-end of the gap. However, if the photoactive group was located on the 5′-end of the gap, p70 subunit was mainly labeled independently of the gap size [49]. These findings are consistent with other data on the more efficient interaction of RPA with the 5′-end region of single-stranded DNA [10]. X-Ray diffraction analysis has shown that single-stranded DNA is bound to the DNA-binding domains A and B of p70 subunit, and the 5′-end of DNA interacts with domain A, whereas domain B is located nearer to the 3′-end [44]. The polarity of the interaction of RPA with single-stranded DNA and the prevalent interaction of p70 with the 5′-end of the gap were confirmed by photoaffinity labeling [49].

Based on these findings, a model was proposed presenting the possible location of RPA in the gaps (Fig. 5, II). In the case of the short gap (9-10 nt) RPA binds to the single-stranded region of the gap interacting with the main DNA-binding domains A and B of subunit, and the RPA conformation is globular, with virtually no contact of p32 subunit with the 3′-end of the primer (Fig. 5, IIa). In the elongated gap, RPA takes the elongated conformation with the easier contacts of p32 with the 3′-end of the primer; however, the interaction of p70 with the single-stranded platform of the gap always dominates, especially on its 5′-end (Fig. 5, IIc).

Note that data on changes in the RPA configuration depending on the length of the protruding region of the template or the gap size were reproduced in studies on

the elongation mechanism of RNA–DNA primers in replicating SV40 chromatin [50, 51]. p32 subunit was shown to make contact with products of the early synthesis, i.e., in the very beginning of the gap filling, whereas p70 subunit became available for contacts with the 3′-end of the growing strand during the later stages of the synthesis [51]. No contacts of p14 subunit in the whole RPA molecule with photoactive DNA of different structure were found both *in vivo* and *in vitro*. This subunit is likely to be covered from contacts with DNA by two other polypeptides of RPA. The interaction of photoactive DNA with individual RPA subunits as chimeric proteins containing a maltose-binding protein was studied, and p14 lacked the DNA-binding activity even at very high concentrations of the protein, although such activity was displayed by p70 and p32 [52]. It seems that p14 plays the role of a polypeptide responsible for the structural organization of the heterotrimer RPA; however, the function of this "contact hinge" in the structure of RPA remains unclear.

Based on all of these results, the interaction of RPA with DNA can be considered as a multistage process initiated by the contact of RPA with a region of 8-10 nt. This binding occurs under the influence of the DNA-binding domains A and B. During this interaction, RPA seems to have a globular shape. In the presence of the more elongated single-stranded region, the RPA conformation is changed, providing for the interaction with DNA of the domain C of p70 and then of DNA-binding domain D, which has been detected in p32. This type of RPA binding seems to occur already at the "elongated" conformation in the presence of a single-stranded DNA region of ~30 nt available for recognition. RPA in the complex with the single-stranded DNA region of 30 nt in size has the maximal affinity, and this size of the "DNA-module" can be more often found as a functional unit during DNA transformations with the involvement of RPA [53].

Detailed information about the RPA domains, which have contacts with the 3′-end of the primer, was obtained by combination of photoaffinity labeling with proteolytic hydrolysis of modified RPA [54]. The main products of covalent attachment to the 3′-end of the primer were a fragment of p32 subunit (amino acids 171-290) containing DNA-binding domain D and also the C-terminal domain of this subunit. There were also recorded minor products of labeling of fragments of DNA-binding domain C of p70 subunit, which seemed to be farther from the 3′-end than the DNA-binding domain of p32. Thus, the comprehensive analysis of the modified regions of RPA confirmed the successive binding of domains A, B, C, and D on single-stranded DNA from its 5′- to the 3′-end. Such locations of the DNA-binding domains seem to simplify the RPA displacement on the filling of single-stranded gaps during the DNA synthesis by DNA polymerases.

OTHER DNA POLYMERASES AND REPLICATION FACTORS

DNA polymerase β. DNA polymerase β is the least in size and the simplest in structure of eucaryotic DNA polymerases [2]. This enzyme is sufficiently well studied compared to other eucaryotic DNA polymerases. DNA polymerase β is a key enzyme in repair of DNA bases; however, its involvement in replication is also not excluded. This enzyme has been intensively and methodically studied, in particular, by X-ray diffraction analysis [55], and, thus, it is a very good model for understanding the action mechanism of DNA polymerases. X-Ray diffraction analysis resulted in determination of the spatial structure of the DNA polymerase β complex with the primer-template and ddCTP [56] and with double-stranded DNA containing a single-strand break and mononucleotide gap [57] and also of the DNA polymerase β complex with 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) that models damaged DNA regions [58]. Pol β has two enzymatic activities: DNA polymerase (5′→3′-deoxynucleotidyl transferase) and deoxyribophosphate lyase (dRP- or AP-lyase) that by the β-elimination mechanism removes the 5′-terminal apurinic/apyrimidinic residues (AP-sites) delimiting gaps in DNA [59]. Thus, the main function of Pol β seems to be to include a dNMP residue into DNA after the strand cleavage in the damaged region under the action of apurinic/apyrimidinic endonuclease, with subsequent removal of sugar phosphate due to the lyase function of Pol β. It is interesting that the lyase function of Pol β more significantly contributes to base excision repair than the polymerase activity [60]. In the absence of removal of the deoxyribosophosphate residue at the 5′-end of the nick, strand-displacement synthesis is stimulated that switches the repair to the long patch subpathway. On elongated templates, Pol β provides for the distributive polymerization by attaching to the primer one nucleotide residue per cycle of binding to the primer-template complex. However, on filling short gaps in DNA duplexes Pol β can perform the processive synthesis and attach 4-6 nt per cycle [61].

Photoaffinity modification of the dNTP-binding site of rat Pol β was performed in [62] using [γ-^{32}P]-8-azido-ATP and [γ-^{32}P]-5-azido-dUTP in the absence of template and primer. It was earlier shown by these authors that mild proteolysis of DNA polymerase β resulted in production of the N-terminal domain with molecular weight of 8 kD and the C-terminal domain with molecular weight of 31 kD, and by further hydrolysis the latter was cleaved into 6- and 27-kD fragments [63]. In [62] proteolysis of the 27-kD domain additionally resulted in two subdomains with molecular weights of 10 and 12 kD. Peptide mapping of the dNTP-binding site was performed with photoreactive analogs of dATP and dUTP in the presence of appropriate competing natural triphos-

phate. The labeled enzyme was treated with trypsin, and then the peptides were separated and sequenced. The label was distributed into three regions removed from each other in the primary sequence, in amino acid residues 4-40, 142-206, and 263-280 belonging to domains with molecular weights of 8, 10, and 12 kD, respectively. Based on these findings, it was concluded that these three regions were located close together and formed the dNTP-binding site. Results of the peptide mapping are consistent with earlier data on the three-dimensional structure of Pol β. According to these data, three subdomains resulting by the mild hydrolysis of Pol β formed "palm", "finger", and "thumb" [55, 64].

The N-terminal domain with molecular weight of 8 kD was earlier shown to bind single-stranded DNA, whereas the region of 31 kD was unable to do this. To identify amino acid residues of the 8-kD domain of DNA polymerase β involved in the binding of single-stranded DNA, $[^{32}P]p(dT)_{16}$ was directly photoattached to the enzyme [65]. Subsequent trypsinolysis indicated that the cross-links were produced by the fragment containing amino acid residues from 28 to 35, and treatment with V8 proteinase revealed residues from 27 to 58. Thus, Ser30 and His34 residues of the N-terminal lyase domain were modified.

The mechanism of the lyase reaction catalyzed by Pol β was studied by affinity modification and MALDI mass-spectroscopy [66]. The approach included trypsinolysis of covalently bound DNA—protein complexes and their subsequent isolation, peptide mapping, and mass-spectroscopy. The covalent cross-links of the Pol β lyase domain with DNA substrate containing deoxyribophosphate on the 5′-end of the primer in the nick were shown to be produced by Lys72 residue. The authors suggest that the nucleophilic attack by the apurinic/apyrimidinic site during the lyase reaction catalyzed by Pol β occurs with involvement of Lys72 residue of the Pol β lyase domain.

A highly selective modification of the active site of DNA polymerase β by photoreactive analogs of nucleotides containing a photoactive group in the nitrogen base, the so-called catalytically competent labeling, was proposed in works [67, 68]. The essence of this approach was as follows: the photoactive dNTP was preirradiated in the presence of the enzyme and then supplemented with the 5′ radioactively labeled primer in the complex with the template. The nucleotide analog covalently bound to the protein. It was suggested that addition of the template-primer should be associated with incorporation into the 3′-end of the primer only of "catalytically competent" dNMP residues which were covalently bound in the active site or immediately nearby. The conformational mobility of dNTP provided for an elongated linker between the reactive group and the nucleotide moiety of the analog was shown to be important for effective elongation of the primer with the covalently attached dNTP. The nucleotide residue was transferred onto the blunt end of the primer-template only after the covalent fixation of dNTP and was more effective in the presence of Mn^{2+} and not Mg^{2+}. Based on these findings, it was concluded that the binding of dNTP by Pol β and the subsequent nucleotide transfer should occur in the absence of the encoding base of the template. This was consistent with the idea that DNA polymerase β belonged to the same family as terminal deoxynucleotidyl transferase. The base of the template determines the choosing of dNTP by interaction with DNA polymerases as cofactor responsible for formation of a specific complex for selection of dNTP at each stage of the elongation.

The dNTP-binding domain of DNA polymerase β was earlier studied in detail by X-ray diffraction analysis [56]. But by X-ray diffraction only a particular crystalline structure of the enzyme—substrate complex is studied that is unable to represent all conformational changes occurring during the binding and transformation of the substrate. The dNTP-binding site of Pol β was studied by catalytically competent labeling to compare with the data obtained by crystallography. Photoaffinity labeling was performed with a photoreactive analog of dCTP, exo-*N*-[2-(*p*-azidotetrafluorobenzamido)-ethyl]deoxycytidine-5′-triphosphate (FABdCTP) [68]. In the first case the photoreactive analog and radioactively labeled primer annealed with the template were incubated in the presence of DNA polymerase that resulted in incorporation of the analog into the 3′-end of the primer and subsequent affinity labeling of Pol β. In the other case FABdCTP was covalently bound to the protein under the influence of UV, with subsequent addition of the primer-template complex containing the complementary introduced FABdCMP residue. DNA polymerase β could productively bind dNTP in the active site in the absence of the primer-template, but the previous kinetic analysis indicated that Pol β initially bound the primer-template complex and then dNTP. The presence of the common and dideoxy-terminated primer-templates failed to affect the level of covalent attachment of polymerase to the photoreactive analog, and this also suggested the possibility of the template-independent binding of dNTP by this enzyme. Mutations in the Asp276 and Asn279 residues, which are involved in the dNTP binding, were used for studies on the triple complex of DNA polymerase with the primer-template and dNTP. The level of catalytically competent labeling of these mutants was significantly lower than the level of labeling of wild Pol β. These findings show the specificity of attachment of the photoreactive analog to the dNTP-binding site.

But conclusions based on results of the catalytically competent labeling of dNTP-binding sites of DNA polymerases by nucleotide analogs were not quite correct when arylazido derivatives were used because of a possible contribution of long-lived products. These products are generated during the secondary photolysis of one or several products of triplet nitrene. Thus, transformed

analogs of dNTP can be introduced into the primer and give rise to a product of covalent binding of the primer to the protein. The modification by such reagents cannot be prevented by addition of dithiothreitol or excess natural substrate. The irradiated analog was kept for different time before the enzyme, template, and primer were added, and the photolysis products retained their activities in reactions with the protein for at least 1 h. Thus, the protein could be modified not by nucleotide analog but by the reactive primer already after UV-irradiation. Long-lived products of photolysis, which could covalently bind to the protein, were found for perfluoroarylazide- and trifluorochloroazidopyridine-derivatives [69].

Specificity of the photoaffinity labeling of DNA polymerases was increased by a newly developed approach, the so-called photosensitized modification [70]. The radioactively labeled primer synthesized *in situ* in the presence of DNA polymerase, with dNTP analog containing arylazido group as an elongating substrate, was bound to the DNA-binding site of the enzyme, whereas an analog of nucleoside-5′-triphosphate containing a photosensitizer pyrene-dUTP is bound to the dNTP-binding site. During UV-irradiation, the energy was initially absorbed by the photosensitizer and then transferred onto the arylazido group of the primer only in the complex of photoreagent-DNA polymerase-photosensitizer. This approach provided for a highly selective modification of DNA polymerase β in systems reconstructed from purified proteins and on the level of cell and nuclear extracts [71-75].

Proliferating cell nuclear antigen (PCNA). A protein with molecular weight of 36 kD was one of the first isolated replication factors. This protein is necessary for DNA elongation under the action of DNA polymerase δ. Later this protein was found to regulate the cell cycle and was called Proliferating Cell Nuclear Antigen (PCNA). PCNA was first identified as an antigen in an autoimmune disease, systemic lupus erythematosus. This protein was also called cycline because from time to time it was detected during the S-phase of cells. And after the identity of these two proteins had been established, it was called PCNA to escape confusion with factors binding to cycline-dependent protein kinases [8].

The ability of PCNA to move along DNA was analyzed by affinity modification [76]. These experiments were performed using the primed single-stranded circular DNA with 5-[*N*′-*p*-(azidobenzoyl)-3-aminoallyl]dUMP residues located in different positions of the primer. PCNA could be boarded onto DNA only in the case of assemblage of the full complex of RFC(ATP)—PCNA—RPA. In the case of removal of even one component from the reaction mixture or the replacement of ATP by its non-hydrolyzable analog adenosine-5′-O-(3-thiotriphosphate) (ATPγS), DNA did not covalently bind to PCNA and other replication factors. Furthermore, addition of *Pvu*I restrictase to the reaction mixture also completely inhibited the protein cross-linking with DNA, and this suggested that just the closed single-stranded substrate was required for the PCNA-dependent synthesis of DNA. Thus, each of these factors was an indispensable component for assemblage of the productive complex responsible for processivity of DNA polymerase δ. And PCNA as a permanent attendant of DNA polymerase δ is involved in both the long patch base excision repair and nucleotide excision repair. Interaction of PCNA with DNA polymerases β [77] and λ [78] was recently found. Functions of PCNA for these enzymes are insufficiently determined. Therefore, studies in detail on functions of PCNA are of interest. Obviously, the information about this protein obtained by affinity modification is rather limited.

Replication factor C. Replication factor C consists of five subunits with different molecular weights: 140 (RFC140), 40 (RFC40), 38 (RFC38), 37(RFC37), and 36 kD (RFC36) [79]. This protein promotes the boarding of PCNA onto DNA that results in formation of the triple complex RFC—PCNA—DNA polymerase δ, i.e., a holoenzyme responsible for the processive synthesis of DNA.

The interaction of RFC with the primer-template complex was studied using a synthetic hairpin-producing DNA. Such a hairpin with a protruding 5′-end of the "template" was synthesized to study the mechanism of recognition of the DNA primer by DNA polymerases and the attendant proteins [80] (Fig. 6). To identify the DNA-binding subunit of RFC from HeLa cells, a residue of 5-bromo-2′-deoxyuridine was introduced into the 3′-end of the hairpin prepared by annealing. The addition of RFC and subsequent UV-irradiation of the reaction mixture resulted in the covalent binding only of the largest sub-

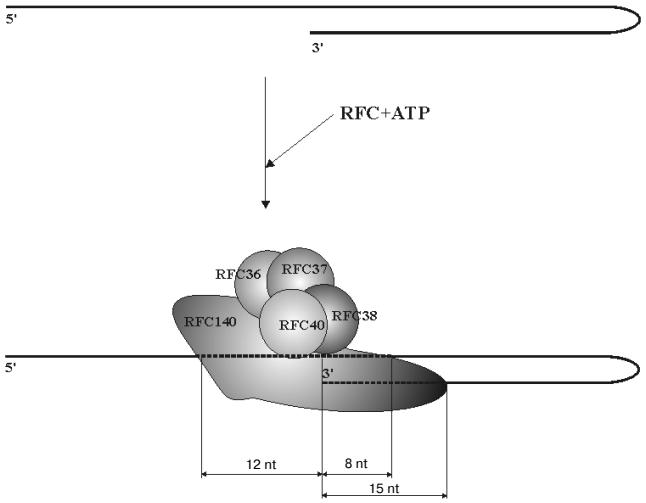

Fig. 6. Model of interaction of RFC with DNA hairpin containing protruding 5′-end [80]. In the presence of ATP RFC efficiently interacts with the protruding end of the hairpin and covers 12 nt of single-stranded DNA, 8 nt of the template, and 15 nt of the primer in the duplex.

unit, RFC140, to the 3′-end of the primer. By affinity modification combined with foot-printing RFC was shown to efficiently bind to the 3′-end of the primer and cover 12 nt of the free end of the template, 8 nt of the template complementary bound to the primer, and 15 nt of the primer (Fig. 6). The ATP-binding subunit of replication factor C was identified by direct photocross-linking with [α-^{32}P]dATP. The RFC37 polypeptide was found to be responsible for the binding to ATP. The subsequent cloning and expression of individual RFC subunits indicated that RFC140 really was the main DNA-binding subunit, whereas RFC36 was responsible for the binding and hydrolysis of ATP [79].

The interaction of mouse RFC with 3′- and 5′-ends of the DNA primer was also studied using photoaffinity modification [81]. DNA duplexes with protruding 3′- or 5′-ends of the template and with gaps of 30 nt in length were used as photoreactive replication intermediates. It was clearly shown that RFC mainly interacted with the 5′-end of the primer and not with its 3′-end. The DNA-binding site of RFC was located on the RFC140 subunit, because just this subunit was detected to be covalently bound to the 5′-end of the primer, and this confirmed its DNA-binding function. Based on the data of [81] together with the earlier published quantitative determinations of the RFC interactions with various DNA structures [82], a model was suggested which described the switching of the DNA synthesis from DNA polymerase α to the synthesis catalyzed by PCNA-dependent DNA polymerases (Fig. 7). According to this model, Pol α initiates the primer synthesis on the single-stranded DNA covered with RPA molecules. RFC binds to the 5′-end of the just synthesized primer (Fig. 7a) and inhibits the DNA synthesis catalyzed by Pol α when the latter has synthesized the RNA–DNA primer of 30 nt in length (Fig. 7b). At this stage, replication factor C seems to promote the boarding of PCNA onto DNA. This results in displacement of Pol α from the 3′-end of the growing strand, and the synthesis is continued with involvement of DNA polymerases δ or ε. It is likely that differences in the data and conclusions about the type of the RFC interaction with DNA are caused by the absence of the free 5′-end in the DNA structure used in [80].

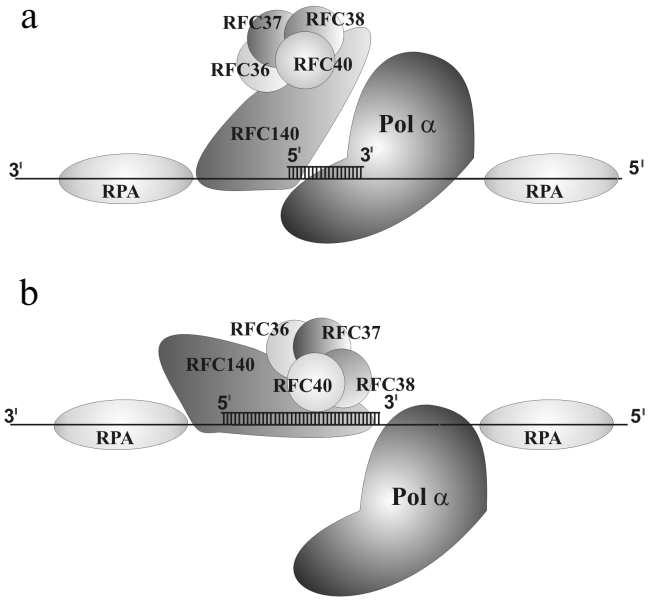

Fig. 7. Regulatory mechanism of DNA replication by replication factor C [81]: a) DNA polymerase α-primase synthesizes the RNA–DNA primer; b) RFC displaces Pol α on the elongation of the RNA–DNA primer to 30-40 nucleotides.

Affinity modification of replication proteins in multicomponent systems. *Affinity modification of proteins of nuclear extract from Physarum polycephalum.* Derivatives of pyrimidine nucleoside-5′-triphosphates containing bulky aromatic substituents in the heterocyclic base were earlier shown to be efficient substrates for all DNA polymerases studied. This feature was used for synthesis of primers with a photoreactive group on the 3′-end to be used for affinity modification of DNA polymerases and replication factors in nuclear extracts. This approach was used to identify components of the replication complex in the nuclear extract from *Physarum polycephalum* [14] that was supplemented with activated DNA and photoactive analogs of dCTP and [α-^{32}P]dATP incorporated into the growing DNA strand due to activity of DNA polymerases of the cells. The reaction mixture was UV-irradiated and treated with nucleases to remove elongated regions of the covalently attached DNA. Using antibodies, the labeled products were shown to contain proteins similar to eucaryotic DNA polymerases α, β, δ, and ε, and also to replication factors PCNA, RPA, and RFC.

The affinity modification technique used with these reagents is sufficiently sensitive for identification of DNA polymerases and replication factors in the nuclear extract. DNA Polymerases α, δ, and ε from *Physarum polycephalum* were found to be similar to DNA polymerases of higher eucaryotes. However, the proteins corresponding to eucaryotic replication factors PCNA and RPA had molecular weights unusual for proteins of this class. Moreover, it has been confirmed that the plasmodium *Physarum polycephalum* has complexes of DNA polymerases with β-poly(L-malate) which is a specific polymer of this organism responsible for the DNA regulation in it [83].

Affinity modification of proteins of cell extract from mouse fibroblasts. Photoaffinity modification was successfully used also to study the base excision repair complex using photoreactive DNA synthesized *in situ* in cell extracts from mouse fibroblasts [84, 85]. Interaction of proteins involved in base excision repair with DNA intermediates produced during this process was studied using photoreactive DNAs prepared *in situ* from ^{32}P-labeled oligonucleotide duplexes containing the apurinic/apyri-

midinic site. Apurinic/apyrimidinic endonuclease of the cell extract cuts DNA in this region, resulting in generation of a nick with hydroxyl and sugar-phosphate group on the 3′- and 5′-ends, respectively. Then the dCMP residue carrying the photoreactive arylazido group was introduced into DNA by cellular DNA polymerases using photoreactive analog of dCTP, which was added into the extract. The photoaffinity labeling under the influence of UV-irradiation resulted in the covalent attachment of only six proteins of the cell extract to the *in situ* produced photoreactive nick. Four proteins were identified as poly(ADP-ribose)polymerase, flap endonuclease 1, DNA polymerase β, and apurinic/apyrimidinic endonuclease, thus interaction of the protein with DNA intermediates of the base excision repair was specific. This work has clearly demonstrated that the affinity modification technique is promising for detection of components of DNA replication and repair complexes.

DNA replication is one of key processes responsible for the stability of genetic information in the cell. DNA replication is *in vivo* performed due to ordered and highly organized actions of numerous DNA polymerases and protein factors which form a "replisome" located in the "replication fabric" of cell nuclei. At present, the operational mode of the replication machine is unclear in detail. The replication fork seems to be a very dynamic structure to provide for the repeated switching from the initiating DNA polymerase α to DNA polymerase δ with involvement of many protein factors. Thus, the protein complex on the lagging strand can initiate, elongate, and perform the processing of Okazaki fragments. It is coming more obvious that DNA replication cannot be considered as a process separate from DNA repair. The *in vitro* reconstructions of repair systems of nucleotides and bases have shown that many of protein factors of DNA replication are involved in repair of both nucleotides and bases [5]. Involvement of about 23 different proteins in the synthesis of Okazaki fragments was recently established [5]. This system seems to be organized in a so-called "replisome" due to protein—protein interactions. On the other hand, such a complex, possibly, does not exist, and these functions are realized by dynamic particular complexes formed on DNA depending on its structure during the transformations. The second hypothesis is now more verified. At least, such dynamic structures are *in vivo* found for systems of nucleotide excision repair [86]. Organization of specific protein structures during transformations of DNA can provide for both the higher dynamics of the system and use of the same factors during DNA replication and repair. X-Ray diffraction analysis and other instrumental approaches are unlikely to be suitable for studies on these systems. However, affinity modification can use virtually every photoreactive intermediate of DNA replication and repair for covalent fixation and subsequent analysis of proteins, even in the case of insufficient stability of their complexes with DNA. This approach seems promising for systems reconstructed from DNA-metabolizing individual components and also on the level of cell and nuclear extracts. Progress in identification of new proteins by MALDI mass-spectroscopy [87] seems promising for discovery of new intermediates and factors involved in DNA replication. The combined use of chemical approaches and, for example, of confocal fluorescent spectroscopy [88] seems to be of help in describing the behavior of supramolecular machines both *in vitro* and *in vivo*.

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REFERENCES

1. Sinha, N. K., Morris, C. F., and Alberts, B. M. (1980) *J. Biol. Chem.*, **255**, 4290-4293.
2. Hubscher, U., Maga, G., and Spadari, S. (2002) *Annu. Rev. Biochem.*, **71**, 133-163.
3. Ayyagari, R., Gomes, X. V., Gordenin, D. A., and Burgers, P. M. (2003) *J. Biol. Chem.*, **278**, 1618-1625.
4. Maga, G., Villani, G., Tillement, V., Stucki, M., Locatelli, G. A., Frouin, I., Spadari, S., and Hubscher, U. (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 14298-14303.
5. Hubscher, U., and Seo, Y. S. (2001) *Mol. Cell*, **12**, 149-157.
6. Tom, S., Henricksen, L. A., Park, M. S., and Bambara, R. A. (2001) *J. Biol. Chem.*, **276**, 24817-24825.
7. Mossi, R., Ferrari, E., and Hubscher, U. (1998) *J. Biol. Chem.*, **273**, 14322-14330.
8. Tsurimoto, T. (1998) *Biochim. Biophys. Acta*, **1443**, 23-39.
9. Yuzhakov, A., Kelman, Z., Hurwitz, J., and O'Donnell, M. (1999) *EMBO J.*, **18**, 6189-6199.
10. Iftode, C., Daniely, Y., and Borowiec, J. A. (1999) *Crit. Rev. Biochem. Mol. Biol.*, **34**, 141-180.
11. Hubscher, U., Nasheuer, H.-P., and Syvaoja, J. E. (2000) *Trends Biochem. Sci.*, **25**, 143-147.
12. Kurganov, B. I., Nagradova, N. K., and Lavrik, O. I. (1996) *Chemical Modification of Enzymes*, Nova Science Publishers, N.Y.
13. Graifer, D. M., and Karpova, G. G. (2001) *Mol. Biol. (Moscow)*, **35**, 584-596.
14. Doerhoefer, S., Khodyreva, S., Safronov, I. V., Wlassoff, W. A., Anarbaev, R., Lavrik, O. I., and Holler, E. (1998) *Microbiology*, **144**, 3181-3193.
15. Lavrik, O. I., and Nevinsky, G. A. (1988) *Affinity Modification of Enzymes: Problems and Prospects* [in Russian], VINITI, Moscow.
16. Knorre, D. G., Kudryashova, N. V., and Lavrik, O. I. (1998) *Usp. Khim.*, **67**, 486-502.
17. Sergiev, P. V., Dontsova, O. A., and Bogdanov, A. A. (2001) *Mol. Biol. (Moscow)*, **35**, 559-583.
18. Mikhailov, V. S. (1999) *Mol. Biol. (Moscow)*, **33**, 567-580.
19. Anarbaev, R. O., Lokhova, I. A., and Lavrik, O. I. (1999) *Mol. Biol. (Moscow)*, **33**, 740-749.
20. Lavrik, O. I., and Nevinsky, G. A. (1989) *Biokhimiya*, **54**, 757-764.

21. Kolocheva, T. I., Nevinsky, G. A., Levina, A. S., Khomov, V. V., and Lavrik, O. I. (1991) *J. Biomol. Struct. Dyn.*, **9**, 169-186.
22. Nevinsky, G. A., Veniaminova, A. G., Levina, A. S., Podust, V. N., Lavrik, O. I., and Holler, E. (1990) *Biochemistry*, **29**, 1200-1207.
23. Nevinsky, G. A., Levina, A. S., Podust, V. N., and Lavrik, O. I. (1987) *Bioorg. Khim.*, **13**, 58-68.
24. Misra, H. S., Pandey, P. K., Modak, M. J., Vinayak, R., and Pandey, V. N. (1998) *Biochemistry*, **37**, 1917-1925.
25. Abboud, M. M., Sim, W. J., Loeb, L. A., and Mildvan, A. S. (1978) *J. Biol. Chem.*, **253**, 3415-3421.
26. Podust, V. N., Korobeinicheva, T. O., Nevinsky, G. A., Levina, A. S., and Lavrik, O. I. (1990) *Mol. Biol. Rep.*, **14**, 247-249.
27. Podust, V. N., Korobeinicheva, T. O., Nevinsky, G. A., Rikhtern, V. A., Abramova, T. I., and Lavrik, O. I. (1990) *Bioorg. Khim.*, **16**, 226-235.
28. Diffley, J. F. X. (1988) *J. Biol. Chem.*, **263**, 14669-14677.
29. Diffley, J. F. X. (1988) *J. Biol. Chem.*, **263**, 19126-19131.
30. Izuta, S., and Saneyoshi, M. (1989) *Biochem. Biophys. Res. Commun.*, **161**, 514-519.
31. Doronin, S. V., Dobrikov, M. I., and Lavrik, O. I. (1992) *FEBS Lett.*, **313**, 31-33.
32. Fioani, M., Linder, A. J., Hartmann, G. R., Lucchini, G., and Plevani, P. (1989) *J. Biol. Chem.*, **264**, 2189-2194.
33. Holmes, A. M., Cheriathundam, E., Bollum, F. J., and Chang, L. M. (1986) *J. Biol. Chem.*, **261**, 11924-11930.
34. Zakharova, O. D., Podust, V. N., Mustaev, A. A., Anarbaev, R. O., and Lavrik, O. I. (1995) *Biochimie*, **77**, 699-702.
35. Copeland, W. C., and Wang, T. S. F. (1993) *J. Biol. Chem.*, **268**, 26179-26189.
36. Sheaff, R., and Kuchta, R. D. (1993) *Biochemistry*, **32**, 3027-3037.
37. Arezi, B., Kirk, B. W., Copeland, W. C., and Kuchta, R. D. (1999) *Biochemistry*, **38**, 12899-12907.
38. Hughes, P., Tratner, I., Ducoux, M., Piard, K., and Baldacci, G. (1999) *Nucleic Acids Res.*, **27**, 2108-2114.
39. Liu, L., Mo, J., Rodriguez-Belmonte, E. M., and Lee, M. Y. (2000) *J. Biol. Chem.*, **275**, 18739-18744.
40. Podust, V. N., Chang, L.-S., Ott, R., Dianov, G., and Fanning, E. (2002) *J. Biol. Chem.*, **277**, 3894-3901.
41. Reems, J. A., Wood, S., and McHenry, C. S. (1995) *J. Biol. Chem.*, **270**, 5606-5613.
42. Mozzherin, D. J., Tan, C. K., Downey, K. M., and Fisher, P. A. (1999) *J. Biol. Chem.*, **274**, 19862-19867.
43. Philipova, D., Mullen, J. R., Maniar, H. S., Lu, J., Gu, C., and Brill, S. J. (1996) *Genes Dev.*, **10**, 2222-2233.
44. Bochkareva, E., Korolev, S., Lees-Miller, S. P., and Bochkarev, A. (2002) *EMBO J.*, **21**, 1855-1863.
45. Wold, M. S. (1997) *Annu. Rev. Biochem.*, **66**, 61-92.
46. Lavrik, O. I., Nasheuer, H.-P., Weisshart, K., Wold, M. S., Prasad, R., Beard, W. A., Wilson, S. H., and Favre, A. (1998) *Nucleic Acids Res.*, **26**, 602-607.
47. Lavrik, O. I., Kolpashchikov, D. M., Weisshart, K., Nasheuer, H.-P., Khodyreva, S. N., and Favre, A. (1999) *Nucleic Acids Res.*, **27**, 4235-4240.
48. Lavrik, O. I., Kolpashchikov, D. M., Nasheuer, H.-P., Weisshart, K., and Favre, A. (1998) *FEBS Lett.*, **441**, 186-190.
49. Kolpashchikov, D. M., Khodyreva, S. N., Khlimankov, D. Y., Wold, M. S., Favre, A., and Lavrik, O. I. (2001) *Nucleic Acids Res.*, **29**, 373-379.
50. Mass, G., Nethanel, T., and Kaufmann, G. (1998) *Mol. Cell. Biol.*, **18**, 6399-6407.
51. Mass, G., Nethanel, T., Lavrik, O. I., Wold, M. S., and Kaufmann, G. (2001) *Nucleic Acids Res.*, **29**, 3892-3899.
52. Kolpashchikov, D. M., Weisshart, K., Nasheuer, H.-P., Khodyreva, S. N., Fanning, E., Favre, A., and Lavrik, O. I. (1999) *FEBS Lett.*, **450**, 131-134.
53. Maga, G., Villani, G., Tillement, V., Stucki, M., Locatelli, G. A., Frouin, I., Spadari, S., and Hubscher, U. (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 14298-14303.
54. Pestryakov, P. E., Weisshart, K., Schlott, B., Khodyreva, S. N., Kremmer, E., Grosse, F., Lavrik, O. I., and Nasheuer, H.-P. (2003) *J. Biol. Chem.*, **278**, 17515-17524.
55. Sawaya, M. R., Pelletier, H., Kumar, A., Wilson, S. H., and Kraut, J. (1994) *Science*, **264**, 1930-1935.
56. Pelletier, H., Sawaya, M. J., Kumar, A., Wilson, S. H., and Kraut, J. (1994) *Science*, **264**, 1891-1903.
57. Sawaya, M. M., Prasad, R., Wilson, S. H., Kraut, J., and Pelletier, H. H. (1997) *Biochemistry*, **36**, 11205-11215.
58. Krahn, J. M., Beard, W. A., Miller, H., Grollman, A. P., and Wilson, S. H. (2003) *Structure (Camb.)*, **11**, 121-127.
59. Matsumoto, Y., and Kim, K. (1995) *Science*, **269**, 699-702.
60. Sobol, R. W., Prasad, R., Evenski, A., Baker, A., Yang, X. P., Horton, J. K., and Wilson, S. H. (2000) *Nature*, **405**, 807-810.
61. Prasad, R., Beard, W. A., and Wilson, S. H. (1994) *J. Biol. Chem.*, **269**, 18096-18101.
62. Srivastava, D. K., Evans, R. K., Kumar, A., Beard, W. A., and Wilson, S. H. (1996) *Biochemistry*, **35**, 3728-3734.
63. Kumar, A., Widen, S. G., Williams, K. R., Kedar, P., Karpel, R. L., and Wilson, S. H. (1990) *J. Biol. Chem.*, **265**, 2124-2131.
64. Davies, J. F., Almassy, R. J., Hostomska, Z., Ferre, R. A., and Hostomsky, Z. (1994) *Cell*, **76**, 1123-1133.
65. Prasad, R., Kumar, A., Widen, S. G., Casas-Finet, J. R., and Wilson, S. H. (1993) *J. Biol. Chem.*, **268**, 22746-22755.
66. Deterding, L. J., Prasad, R., Mullen, G. P., Wilson, S. H., and Tomer, K. B. (2000) *J. Biol. Chem.*, **275**, 10463-10471.
67. Lavrik, O. I., Zakharenko, A. L., Prasad, R., Vlasov, V. A., Bogachev, V. S., and Favre, A. (1998) *Mol. Biol. (Moscow)*, **32**, 621-628.
68. 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.
69. Zakharenko, A. L. (2001) *Interaction of DNA Polymerases with dNTP and Their Photoreactive Analogs*: Author's abstract of Candidate's dissertation (Chemistry) [in Russian], Novosibirsk Institute of Bioorganic Chemistry, Novosibirsk.
70. Kolpashchikov, D. M., Rechkunova, N. I., Dobrikov, M. I., Khodyreva, S. N., Lebedeva, N. A., and Lavrik, O. I. (1999) *FEBS Lett.*, **448**, 141-144.
71. Lavrik, O. I., Kolpashchikov, D. M., Prasad, R., Sobol, R. W., and Wilson, S. H. (2002) *Nucleic Acids Res.*, **30**, e73.
72. Rechkunova, N. I., Kolpashchikov, D. M., Lebedeva, N. A., Petruseva, I. O., Dobrikov, M. I., Degtyarev, S. Kh., and Lavrik, O. I. (2000) *Biochemistry (Moscow)*, **65**, 244-249.
73. Lebedeva, N. A., Kolpashchikov, D. M., Rechkunova, N. I., Khodyreva, S. N., and Lavrik, O. I. (2001) *Biochem. Biophys. Res. Commun.*, **287**, 530-535.

74. Lebedeva, N. A., Kolpashchikov, D. M., Rechkunova, N. I., Khodyreva, S. N., and Lavrik, O. I. (2002) *Biochemistry (Moscow)*, **67**, 807-814.
75. Lebedeva, N. A., Rechkunova, N. I., Dezhurov, S. V., Khodyreva, S. N., Favre, A., and Lavrik, O. I. (2003) *Biochemistry (Moscow)*, **68**, 476-481.
76. Tinker, R. L., Kassavetis, G. A., and Geiduschek, E. P. (1994) *EMBO J.*, **13**, 5330-5337.
77. Kedar, P. S., Kim, S. J., Robertson, A., Hou, E., Prasad, R., Horton, J. K., and Wilson, S. H. (2002) *J. Biol. Chem.*, **277**, 31115-31123.
78. Maga, G., Villani, G., Ramadan, K., Shevelev, I., Le Gac, N. T., Blanco, L., Blanca, G., Spadari, S., and Hubscher, U. (2002) *J. Biol. Chem.*, **277**, 48434-48440.
79. Mossi, R., and Hubscher, U. (1998) *Eur. J. Biochem.*, **254**, 209-216.
80. Tsurimoto, T., and Stillman, B. (1991) *J. Biol. Chem.*, **266**, 1950-1960.
81. Kolpashchikov, D. M., Hughes, P., Favre, A., Baldacci, G., and Lavrik, O. I. (2001) *J. Mol. Recogn.*, **14**, 239-244.
82. Maga, G., Stucki, M., Spadari, S., and Hubscher, U. (2000) *J. Mol. Biol.*, **295**, 791-801.
83. Doerhoefer, S., Windisch, C., Angerer, B., Lavrik, O. I., Lee, B. S., and Holler, E. (2002) *Eur. J. Biochem.*, **269**, 1253-1258.
84. Lavrik, O. I., Prasad, R., Sobol, R. W., Horton, J. K., Ackerman, E. J., and Wilson, S. H. (2001) *J. Biol. Chem.*, **276**, 25541-25548.
85. Prasad, R., Lavrik, O. I., Kim, S. J., Kedar, P., Yang, X. P., Vande Berg, B. J., and Wilson, S. H. (2001) *J. Biol. Chem.*, **276**, 32411-32414.
86. Volker, M., Mone, M. J., Karmakar, P., van Hoffen, A., Schul, W., Vermeulen, W., Hoeijmakers, J. H., van Driel, R., van Zeeland, A. A., and Mullenders, L. H. (2001) *Mol. Cell*, **8**, 213-224.
87. Steen, H., and Jensen, O. N. (2002) *Mass Spectrom. Rev.*, **21**, 163-182.
88. Segers-Nolten, G. M., Wyman, C., Wijgers, N., Vermeulen, W., Lenferink, A. T., Hoeijmakers, J. H., Greve, J., and Otto, C. (2002) *Nucleic Acids Res.*, **30**, 4720-4727.