Volume 313, number 1, 31–33 FEBS 11753 November 1992

Photoaffinity labeling of DNA polymerase α DNA primase complex based on the catalytic competence of a dNTP reactive analog

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Received 17 September 1992

FABdCTP was found to be a substrate of DNA polymerization catalyzed by a DNA polymerase α–DNA primase complex on the 5′-GTGAGTAAGTGGAGTTTGGCACGAT-3′ template and 3′-CTCAAACCGT-5′ primer. After complete primer extension in the presence of FABdCTP under UV-irradiation of the reaction mixture, 70% of the template was covalently linked to the primer. Labeling of the 165 kDa subunit of the DNA polymerase α, 59 kDa and 49 kDa subunits of the DNA primase and an unknown protein with apparent molecular weight of 31 kDa was observed. By another way of protein labeling FABdCTP was covalently bound to the subunits of the enzyme under UV irradiation and then this moiety was introduced into the 3′-end of the 5′-[^{32}P]primer by the catalytic activity of DNA polymerase or DNA primase. In this case covalent labeling of the 165 kDa, 49 kDa and 31 kDa subunits was observed.

DNA polymerase α; Photoaffinity modification

1. INTRODUCTION

A complicated network of interacting protein factors and enzymes is required for DNA replication [1]. Reconstitution of the SV40 DNA replication system demands at least two DNA polymerases (α and δ) and a number of replication factors (RF-A, RF-C, PCNA, SV40 large tumor antigen and topoisomerase II) [2–4]. All these proteins act cooperatively in replication fork. A multiprotein complex with molecular weight of 640 kDa has been isolated from the HeLa cells. This complex contains DNA polymerase α, DNA primase, 3′-5′ exonuclease, Ap_4A binding protein and primer recognition accessory proteins C2 and C3 [5,6]. It is very likely that specific protein–protein interactions of components in this multiprotein complex determine the specificity and efficiency of DNA replication.

We believe that for identification of the functional relations among proteins in the replicative machinery new specific tools are required. Here we have used base-substituted dNTP with highly reactive chemical groups reacting both with proteins and DNA. These analogs are substrates of the DNA polymerase.

2. MATERIALS AND METHODS

Immunoaffinity-purified DNA polymerase α-DNA primase from human placenta with specific activity of 30,000 U/mg was obtained as described in [7]. Polynucleotide kinase and dNTP were from NIKTI BAV (Russia), $MgCl_2$ from Merck, [γ-^{32}P]ATP (spec. act. > 100 PBq/mol) from Izotop (Russia), other reagents were of analytical grade. Synthesis of the 5′-GTGAGTAAGTGGAGTTTGGCACGAT-3′ template and the 3′-CTCAAACCGT-5′ primer was performed according to [8]. Synthesis of exo-*N*-[2-(*p*-azidotetrafluorobenzamido)ethyl]-deoxycytidine-5′-triphosphate (FABdCTP, Fig. 1) was described in [9]. ^{32}P-labeled oligonucleotides with specific activity of 1.3×10^{15} cpm/mol was obtained using polynucleotide kinase.

The reaction mixture for the DNA polymerase assay (10–30 μl) contained 50 mM Tris-HCl buffer (pH 7.5), 0.04 M KCl, 0.005 M $MgCl_2$, 1 μM 5′-GTGAGTAAGTGGAGTTTGGCACGAT-3′ template, 1 μM 5′-^{32}P-labeled 3′-CTCAAACCGT-5′ primer, 10 μM FABdCTP. The reaction of DNA synthesis was carried out at 30° C and started by the addition of the 30 unit act. of the DNA polymerase α–DNA primase complex. Products of the primer extension were analyzed by electrophoresis in 20% acrylamide gels in the presence of 8 M urea.

Modification of DNA polymerase α and DNA was done by irradiation of the reaction mixture by filtered light of a high-pressure mercury lamp (energy 2.5 $J \cdot cm^{-2} \cdot c^{-1}$) for 1.5 min at 303–365 nm. After UV-irradiation 1% SDS was added. The assay mixture was analyzed by electrophoresis in 10% acrylamide gels according to Laemmli [10].

3. RESULTS AND DISCUSSION

It was shown earlier that FABdCTP is a substrate for the DNA polymerization reaction catalyzed by HIV reverse transcriptase on M13mp18 and M13mp10 DNA templates [9]. In the present work we found that DNA polymerase α uses FABdCTP instead of dCTP (Fig. 2). The efficiency of the primer extension was the same as when dCTP at the same concentration (10 μM) was used.

After complete primer extension and UV light irradiation of the reaction mixture, the primer was covalently

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Abbreviation: FABdCTP, exo-*N*-[2-(*p*-azidotetrafluorobenzamido)-ethyl]-deoxycytidine-5′-triphosphate.

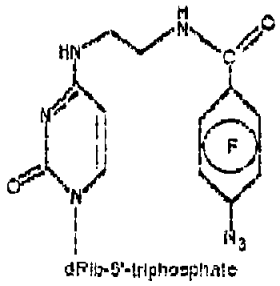

Fig. 1. Structural formula of FABdCTP.

attached to the template which caused a shift-up of the radioactive band on the electrophoresis (Fig. 2). As a result 70% of template was modified.

After irradiation of the enzyme with the FABdCTP followed by incubation of the mixture with the template and 5'-[^{32}P]primer, labeling of the 165 kDa, 49 kDa and 31 kDa subunits was observed (Fig. 3). All of these proteins were not labeled when using UV-light preirradiated FABdCTP. These observations strongly support the suggestion that the FABdCTP moiety covalently bound with the proteins was included into the primer chain through the catalytic activity of the DNA polymerase α or DNA primase. Labeling of the 59 kDa subunit of the DNA primase was found when the enzyme was UV-irradiated with completely elongated primer (Fig. 3). In the present case the reason for labeling of the enzyme subunits is association–dissociation of the template–primer duplex with the enzyme. Labeling of

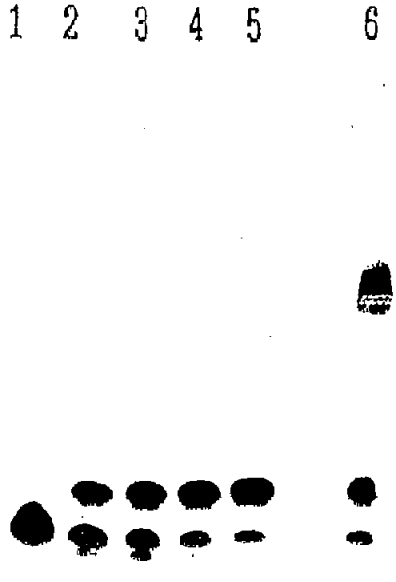

Fig. 2. Kinetic elongation of the 3'-CTCAAACCGT-5' primer with the 5'-GTGAGTAAGTGGAGTTTGGCACGAT-3' template by DNA polymerase α–DNA primase in the presence of FABdCTP: lane 1, 0 min; lane 2, 30 min; lane 3, 60 min; lane 4, 120 min; lane 5, 180 min; lane 6, electrophoretic patterns after preincubation for 180 min with template, 5'-^{32}P-labeled primer and FABdCTP with DNA polymerase α–DNA primase followed by UV light irradiation of the reaction mixture.

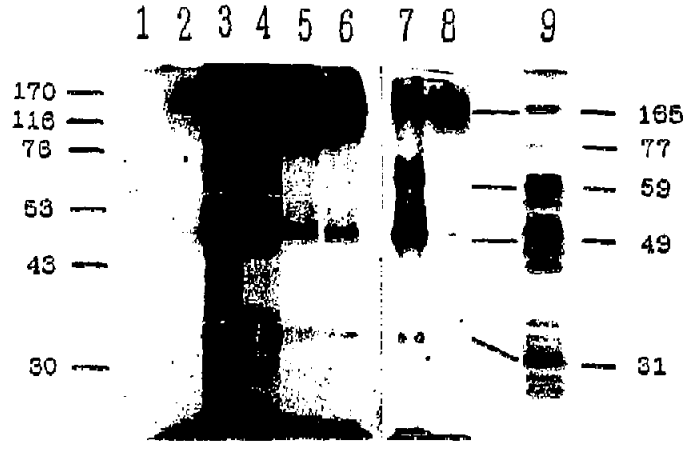

Fig. 3. Cross-linking of the DNA polymerase α–DNA primase with the 5'-^{32}P-labeled primer in the presence of FABdCTP: lane 1, DNA polymerase preincubated with template, 5'-[^{32}P]primer, and FABdCTP; lane 2, DNA polymerase preincubated with the template, 5'-[^{32}P]primer, and UV light irradiated FABdCTP; lane 3, DNA polymerase incubated with the template, 5'-[^{32}P]primer, and FABdCTP followed by UV light irradiation; lane 4, DNA polymerase irradiated with the template, 5'-[^{32}P]primer, and FABdCTP and subsequent incubation; lane 5, DNA polymerase irradiated with the template and FABdCTP with the subsequent incubation in the presence of the 5'-[^{32}P]primer; lane 6, DNA polymerase irradiated with FABdCTP and with subsequent incubation in the presence of the template and 5'-[^{32}P]primer. Lanes 7 and 8 are the same as lanes 3 and 6, respectively, with a shorter exposure. Lane 9, silver-stained DNA polymerase α–DNA primase. Molecular weight markers are on the left and apparent molecular weight of the proteins found in the preparation of the DNA polymerase α–DNA primase are shown on the right. The incubation time was 3 h.

the 59 kDa subunit was not detected and labeling of the 49 kDa subunit decreased ten times at short time of preincubation of the enzyme with the template, [^{32}P]primer and FABdCTP before UV light irradiation (less than 15 s, when less than 1% of the primer was extended). In this case labeling of the enzyme can be effected in two ways. FABdCTP moiety bound to the proteins is included into the primer chain through the catalytic activity of DNA polymerase α or DNA primase (catalytically competent labeling). Alternatively, the extended primer is covalently bound to the 49 kDa subunit without dissociating from the enzyme. Labeling of the 49 kDa subunit decreased another ten times when the enzyme was irradiated with FABdCTP in the presence template and subsequent incubating of the mixture with the 5'-[^{32}P]primer. We suggest that catalytically competent labeling of the primase occurs with relatively low efficiency and the main reason for labeling of the 49 kDa subunit in the previous case is covalent binding of the primer synthesized in situ by the DNA polymerase. Based on this we assume that, unlike the 59 kDa subunit, the primase 49 kDa subunit should have a contact with the 165 kDa subunit DNA polymerase near site binding of the 3'-end of the primer.

Labeling of the 165 kDa subunit does not change dramatically whether the labeling occurs via enzyme

association with the photoreactive primer or by means of catalytically competent labeling (Fig. 3).

Classical preparation of the immuno-purified DNA polymerase α contains a family of catalytic subunits of DNA polymerase from 180 to 120 kDa, a 72 kDa subunit with unknown function and 59 kDa and 49 kDa primase subunits [7]. However, we here isolated a DNA polymerase α which contained an additional low molecular weight protein of 31 kDa. Labeling of this protein was detected in all cases. This is a strong argument against this protein being not an occasional contamination. It can be either a proteolysis product of a DNA polymerase catalytic subunit or a subunit of one of the replication factors associated with DNA polymerase α.

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