5-[3-(*E*)-(4-Azido-2,3,5,6-tetrafluorobenzamido)propenyl-1]-2′-deoxyuridine-5′-triphosphate Substitutes for Thymidine-5′-triphosphate in the Polymerase Chain Reaction

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The DNA targets may be labeled and simultaneously amplified in the polymerase chain reaction (PCR) using a pair of respective primers after elongation with nucleoside-5′-triphosphates carrying photoreactive groups. The amplified DNA may be subsequently photoactivated by irradiation above 300 nm, resulting in photo-cross-linking of the strands. For this goal 5-[3-(*E*)-(4-azido-2,3,5,6-tetrafluorobenzamido)propenyl-1]-, 5-{*N*-[*N*′-(4-azido-2,3,5,6-tetrafluorobenzoyl)-3-aminopropionyl]aminomethyl}-, and 5-{*N*-[*N*′-(2-nitro-5-azidobenzoyl)-3-aminopropionyl]aminomethyl}-2′-deoxyuridine-5′-triphosphate (**VII**, **VIa**, and **VIb**) derivatives have been synthesized. It was found that **VII** is capable of efficiently elongating DNA primers with both Klenow fragment DNA polymerase I and *Thermus aquaticus* DNA polymerase. Thereto, it turned out to provide quantitative incorporation in DNA as revealed by the formation of the full-length amplificate by PCR in the presence of this photoreactive analogue without any dilution with natural dTTP. On the contrary, it was found, that incorporation of **VIa** and **VIb** do not permit further DNA replication.

INTRODUCTION

Nucleoside-5′-triphosphate derivatives carrying a photoreactive aryl azido residue attached to heterocyclic moiety were proposed a few years ago as substrates of the nucleic acid polymerases for the preparation of photoreactive oligonucleotides. UTP and CTP analogues were used for preparation photoreactive transcript, which can be used for affinity labeling of *Escherichia coli* RNA polymerase (*1*, *2*). The dCTP derivative carrying a *p*-perfluoroazido benzoyl residue bound to exocyclic NH_2 group via a $-CH_2CH_2NH-$ spacer was prepared and used for primer extension by a DNA polymerase α-primase complex from human placenta. The duplex obtained was demonstrated to carry out affinity modification of a number of the subunits of the complex (*3*). The same derivative as well as another one, carrying *p*-perfluoroazido benzoyl residue attached to 5-C of dCTP via the spacer $-CH{=}CH-CH_2-NH_2$, was found to be a substrate of the HIV-1 reverse transcriptase, and it was shown that specific modification of DNA template in the complex with the enzyme was carried out (*4*). The primer extension products obtained with 37-meric DNA and M13 DNA templates were found to photo-cross-link with both the enzyme and template DNA (*4*, *5*). Thus, the approach turned out to be promising for the specific labeling of both polymerases and DNA templates. To expand the possibilities of the approach a number of dTTP, dCTP and dATP analogues were prepared as the tools for investigation of nucleic acid—nucleic acid and protein—nucleic acid interactions (*6*).

Recently, we proposed to use this approach to elaborate a new version of the fluorescent DNA probes localization on chromosomes and chromatin (*7*). This version is based upon the elongation of the hybridized probe in the presence of DNA polymerase and a mixture of deoxynucleoside-5′-triphosphate containing a photoreactive analogue of one of the substrates. This procedure permits the subsequent photo-cross-linking of the elongated probe with chromatin, thus making possible the following extensive washing to remove nonspecific signals and, consequently, to achieve higher contrast of the specific one. In our first work, we used a dTTP analogue with 2-nitro-5-azidobenzoyl residue attached to 5-C atom of deoxyuridine-5′-triphosphate via a $-CH_2NHC(O)(CH_2)_2-NH-$ spacer.

Obviously, the efficient incorporation of photoreactive dNTP[1] in the product of DNA replication is one of essential prerequisites for the subsequent efficient cross-linking. The main goal of this work was to prepare the photoreactive nucleoside-5′-triphosphate derivative, which can be efficiently incorporated into DNA primers by DNA polymerases with subsequent cross-linking with the template DNA under irradiation. Since it was shown earlier that, among aryl azides investigated, the *p*-azido perfluorobenzoyl residue is the most reactive toward complementary oligonucleotide chain (*8*, *9*), we have

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[1] Abbreviations: (d)NTP, 2′-deoxyribonucleoside-5′-triphosphates; NTP, nucleoside-5′-triphosphates; DMF, dimethylformamide; TEA, triethylamine; TEAB, triethylammonium bicarbonate; DCC, *N*,*N*′-dicyclohexylcarbodiimide; *Taq* DNA polymerase, *Thermus aquaticus* DNA polymerase.

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prepared new dNTP analogues, carrying this group connected with pyrimidine heterocycle via two different spacers. One of them, 5-[3-(*E*)-(4-azido-2,3,5,6-tetrafluorobenzamido)propenyl-1]-2′-deoxyuridine-5′-triphosphate, turned out to provide quantitative incorporation in DNA as revealed by the formation of the full-length amplificate in the presence of this dU*TP analogue without any dilution with natural dTTP. The amplified DNA subsequently may be photoactivated by irradiation above 300 nm, leading to the photo-cross-linking of the strands. These results indicate that new photoreactive analogue **VII** can be used for in vitro incorporation into DNA probes to label genetic targets on human metaphase chromosome spreads and interphase nuclei after the formation of covalent links between the photoanchoring probes and template DNA.

EXPERIMENTAL PROCEDURES

Materials. *N*,*N*-Dimethylformamide, acetone, acetonitrile, pyridine, and triethylamine were purified and dried according to standard procedures. Triethyl phosphate, *β*-alanine, DCC, *N*-hydroxysuccinimide, and bis-(tri-*n*-butylammonium)pyrophosphate were purchased from Merck (Germany). Phosphoroxychloride, 2′-deoxyuridine, and 2′-deoxyuridine-5′-triphosphate were purchased from Fluka (Switzerland). Dowex 50W X2 was purchased from Serva (Germany). DEAE-cellulose DE-32 was purchased from Whatman (England). Polynucleotide kinase and dNTP were purchased from NIKTI BAV (Russia). DNA polymerase *Thermus aquaticus* was from Biopol (Russia). [^{32}P]-*o*-phosphate was purchased from "Nuclid Trans" (Moscow, Russia). Other reagents were of analytical grade.

General Methods. ^{1}H NMR spectra were recorded on a Bruker WP-200 SY (200 MHz) and on a Bruker AC-200 (200.132 MHz) spectrometers. All ^{1}H chemical shifts are reported relative to TMS as an internal standard. ^{31}P NMR spectra were recorded on a Bruker AC-200 (81.015 MHz) spectrometer, the ^{31}P chemical shifts are reported using an external standard of 85% H_3PO_4. Infrared spectra were recorded on a Specord M80 (Karl Zeiss, Yena). UV absorption spectra were recorded on a Specord M40 (Karl Zeiss, Yena).

Analytical TLC was carried out on silica gel plates (Merck, DC-Alufolien Kieselgel 60 F_{254}) in ethanol/NH_4-OH, 4:1(v/v), (system A); in dioxane/NH_4OH/water, 6:1:4 (v/v), (system B); and in $CHCl_3$/ethanol, 4:1 (v/v), (system C). The compounds containing amino groups were visualized by the ninhydrin reaction (1% ninhydrin, 2% acetic acid in methanol).

Isolation and purification of the dU*TP derivatives were performed by reversed-phase high-performance liquid chromatography using a LiChrosorb RP-18, 10 μm (Merck, FRG), 4.6 × 250 mm column, a Waters 600E chromatograph, and Waters 484 tunable absorbance detector. A linear gradient (2 mL/min) from 0 to 30% of acetonitrile in 0.05 M TEAB (pH 7.5) was used. Analytical anion-exchange microcolumn chromatography was performed on the Milichrom chromatograph (Russia) using a Polisil-SA, 15 μm (Vector, Russia), 2.5 × 30 mm column. A linear gradient (flow rate 50 μL/min) from 0 to 1 M of NaCl in 7 M urea and 0.01 M Na_2HPO_4 (pH 9) was employed.

The oligodeoxyribonucleotides pd(GACCTGCCACTTTG-GCTGAA) and pd(CACAATGCTGCCTTTTCCAA) were prepared by Dr. Galiya A. Maksakova as described (*10*). The oligonucleotide pd(CACAATGCTGCCTTTTCCAA) was 5′-end labeled using T4-polynucleotide kinase, [γ-^{32}P]-ATP (>3000 Ci/mmol). After purification on 20% polyacrylamide gel under denaturing conditions (8 M urea, 89 mM Tris-H_3BO_3, ~40 °C), oligonucleotide [^{32}P]d-(CACAATGCTGCCTTTTCCAA) was used as a primer in PCR experiments.

The amplification of DNA was performed on a DNA Thermal Cycler Manual (Bis, Russia).

thermal cycling profile	
inital denaturation (25−30 cycles)	95 °C, 3 min
denaturation step	95 °C, 36 s
annealing step	55 °C, 60 s
elongation step	70 °C, 2 min

A 50 μL PCR Mastermix contained 1 μg of each primer, 0.01 μg of DNA template (pTBE−51.1 plasmid), 1.5 mM $MgCl_2$, 67 mM Tris-HCl (pH 8.9), 16 mM $(NH_4)_2SO_4$, 0.05% Tween 20, 0.5 mM solution of dATP, dCTP, dGTP, and either dTTP, the photoreactive 2′-deoxyuridine-5′-triphosphate derivative, or mixture of definite amount of both photoreactive dU*TP and dTTP and 0.5 units of *Taq* DNA-polymerase. The reaction products were separated by agarose gel (2%) electrophoresis. The gels were visualized by ethidium bromide staining or autoradiography.

After synthesis of DNA by PCR in the presence of photoreactive dU*TP analogue **VII**, the PCR products were separated in 10% nondenaturating PAAG in the dark. The amplified DNA was extracted from the gel by electroelution and precipitated by addition of 2% $LiClO_4$ in acetone. The precipitate was carefully washed with acetone (2 × 1.2 mL). UV (H_2O) $\lambda_{max,}$ 268 nm; IR (0.02 mm CaF_2 cuvette) $\nu_{max,}$ 2100 ($-N_3$) cm^{-1}.

The M13mp10 phage DNA was isolated from the culture fluid of a recipient K12 x L1 *E. coli* strain. The primer pd(CCCAGTCACGACGT) was labeled at the 5′-position by [γ-^{32}P]ATP with a specific activity of 1500 Ci/mmol (Radioisotop, Russia) using T4-polynucleotide kinase and annealed with the template at the ratio 1:1 under standard conditions [60 °C, 10 min, in 10 mM Tris-HCl buffer (pH 7.9), 5 mM $MgCl_2$, and 1 mM dithiothreitol]. The primer−template complex was purified by gel filtration on a Bio-Gel A (1.5 mesh) microcolumn, prewashed by 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA.

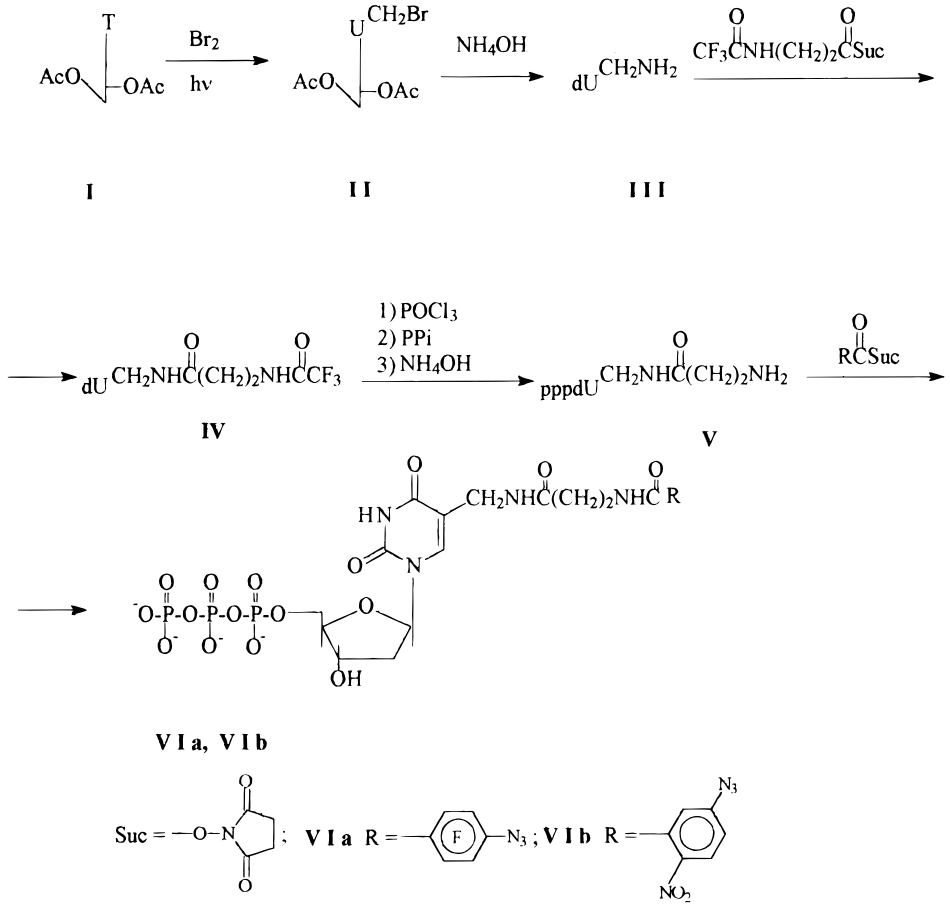
DNA polymerase assays were carried out as described (*11*). The reaction mixture (6 μL) containing 0.2 units of DNA polymerase I Klenow fragment (Promega) or *Taq* DNA polymerase (Biopol, Russia), 10 nM of the primer−template complex, natural substrates (dTTP, dGTP), or the testing compound **VII** was incubated during 15 min at 20 °C for Klenow fragment and 15 min at 72 °C for *Taq* DNA polymerase. The reaction was stopped by addition of 3 μL of formamide containing 0.1% of xylenecyanol, 0.1% of bromophenol blue, and 20 mM EDTA, pH 8.0. The reaction products were separated by electrophoresis in a 20% PAAG under denaturing conditions. The gels were visualized by autoradiography.

N-Hydroxysuccinimide esters of 2-nitro-5-azidobenzoic and 4-azido-2,3,5,6-tetrafluorobenzoic acids were synthesized as described (*12*).

Thymidine monophosphate kinase was isolated from *E. coli* MRE-600 according to the described method (*13*).

Synthesis of 5-{*N*-[3-(trifluoroacetylamino)propionyl]aminomethyl}-2′-deoxyuridine derivative (IV). Compound **IV** was obtained from thymidine in four steps with 68% yield (see Scheme 1) using the method described earlier (*14*): R_f = 0.78 in the system A; UV (H_2O, pH 9) $\lambda_{max,}$ 267 nm (ϵ = 8 800); λ_{min}, 235 nm (ϵ = 3 500). ^{1}H NMR (D_2O): δ 2.35 (m, H2′, 2H), 2.50 (t, J =

Scheme 1



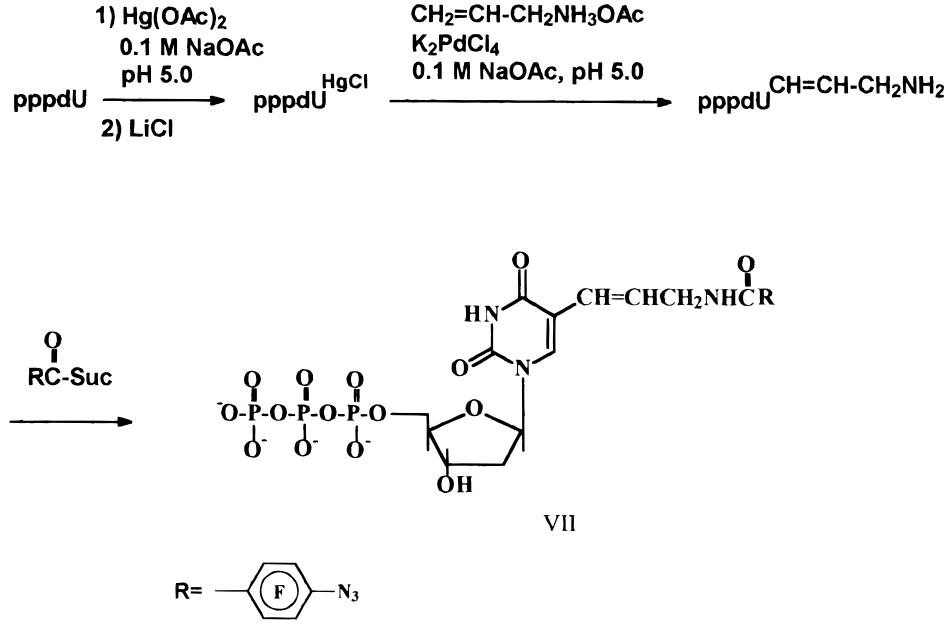
6.0 Hz, H8, 2H), 3.58 (m, H9, 2H), 3.79 (m, H5′, 2H), 4.04 (m, H7, H4′, 3H), 4.45 (m, H3′, 1H), 6.26 (t, J = 6.0 Hz, H1′, 1H), 7.54 (s, H6, 1H).

Synthesis of 5-[*N*-(3-aminopropionyl)aminomethyl]-2′-deoxyuridine-5′-triphosphate derivatives (triethylammonium salt) (V). Compound **V** was obtained from **IV** using the method described earlier for the ATP (*15*). A total of 21 μL (300 μmol) of $POCl_3$ was added to an ice-cooled suspension of **IV** 41.4 mg (100 μmol) in 300 μL of triethyl phosphate. After 16 h at 4 °C, the reaction mixture was treated with a freshly prepared solution of 500 μL of 1 M bis(tri-*n*-butylammonium) pyrophosphate and 100 μL of tri-*n*-butylamine. When the phosphorylation was complete (about 1 min), 10 μL of 1 M triethylammonium bicarbonate was added. After 1 h at room temperature, the reaction mixture was pooled on a rotary evaporator and treated with 10 mL of concentrated aqueous ammonia during 3 h to remove the trifluoroacetic protecting group. The solution was evaporated and diluted with 15 mL of water, and the product was isolated by ion-exchange chromatography on DEAE-cellulose DE-32 (1 × 15 cm) using a gradient of triethylammonium bicarbonate (0.00−0.35 M, pH 7.5). The fraction containing **V** was evaporated and dried in vacuo to give 30 μmol (30%) of **V**: R_f = 0.11 in the system B; UV (H_2O) $\lambda_{max,}$ 268 nm (ϵ = 8800); λ_{min}, 235 nm (ϵ = 3700). ^{31}P NMR (D_2O): δ −21.49 (t, J = 20 Hz, P_β, 1P), −11,16 (d, J = 20 Hz, P_α, 1P), −6.0 (d, J = 20 Hz, P_γ, 1P).

Synthesis of 5-{*N*-[*N*′-(4-azido-2,3,5,6-tetrafluorobenzoyl)-3-aminopropionyl]aminomethyl}-2′-deoxyuridine-5′-triphosphate (lithium salt) (VIa). *N*-Hydroxysuccinimide ester of 4-azido-2,3,5,6-tetrafluorobenzoic acid 4.88 mg (16 μmol) was added to a solution of **V** (4 μmol) in a mixture of DMF (60 μL) and TEA (10 μL). The reaction mixture was kept at room temperature for 4 h and then loaded directly onto a column of DEAE-cellulose DE-32 (1 × 20 cm). A linear gradient (flow rate 0.5 mL/min) from 0.05 to 0.3 M TEAB (pH 7.5) was employed. Fractions containing photoreactive nucleoside-5′-triphosphate were pooled and subjected to reversed-phase HPLC. The product **VIa** was precipitated by addition of 2% $LiClO_4$ in acetone and dried in vacuo. The yield of **VIa** was 87%. The isolated compound was homogeneous as shown by thin-layer and anion-exchange chromatography. R_f = 0.54 in the system B; UV (H_2O) $\lambda_{max,}$ 259 nm (ϵ = 33 900); λ_{min}, 232 nm (ϵ = 10 900). ^{31}P NMR (D_2O): δ −20.9 (t, J = 20 Hz, P_β, 1P), −11.08 (d, J = 20 Hz, P_α, 1P), −5.72 (d, J = 20 Hz, P_γ, 1P). ^{1}H NMR (D_2O): δ 2.43 (dd, J = 6 Hz, J = 2 Hz, H2′, 2H), 2.70 (t, J = 6 Hz, H8, 2H), 3.71 (m, H4′, H9, 3H), 4.30 (m, H3′, H5′, H7, 5H), 6.30 (t, J = 7 Hz, H1′, 1H), 7.90 (s, H6, 1H).

Synthesis of 5-{*N*-[*N*′-(2-nitro-5-azidobenzoyl)-3-aminopropionyl]aminomethyl}-2′-deoxyuridine-5′-triphosphate (lithium salt) (VIb). Compound **VIb** was synthesized from derivative **V** by treatment with *N*-hydroxysuccinimide ester of 2-nitro-5-azidobenzoic acid according to the method described above for the synthesis of **VIa**. The yield of **VIb** was 82%. The isolated compound was homogeneous by thin-layer and anion-exchange chromatography. R_f = 0.55 in the system B; UV (H_2O) λ_{max}, 269 nm (ϵ = 9000), 321 nm (ϵ = 8800); λ_{min}, 245 nm (ϵ = 7900). ^{31}P NMR (D_2O): δ −20.33 (t, J = 20 Hz, P_β, 1P), −10.95 (d, J = 20 Hz, P_α, 1P), −5.10 (d, J = 20 Hz, P_γ, 1P). ^{1}H NMR (D_2O): δ 2.23 (m, H2′, 2H), 2.65 (t, J = 5.2 Hz, H8, 2H), 3.65−3.75 (m, H4′, H9, 3H), 4.16 (m, H3′, H5′, H7, 5H), 6.25 (t, J = 6.5 Hz, H1′, 1H), 7.15 (d, J = 2.3 Hz, H10, 1H), 7.37 (dd, J = 8.9 and 2.3 Hz, H11, 1H), 7.71 (s, H6, 1H), 8.25 (d, H12, 1H).

Scheme 2



Synthesis of 5-[3-(*E*)-(4-Azido-2,3,5,6-tetrafluorobenzamido)-propenyl-1]-2′-deoxyuridine-5′-triphosphate (lithium salt) (VII). Compound **VII** was obtained according to the method of Wlassoff et al. (*6*), described for synthesis of 5-[3-[(*E*)-(2-nitro-5-azidobenzamido)propenyl-1]-2′-deoxyuridine-5′-triphosphate with the use of *N*-hydroxysuccinimide ester of 4-azido-2,3,5,6-tetrafluorobenzoic acid (see Scheme 2). The yield of **VII** (mixture of isomers) was 90%. The separation of isomers was carried out by reversed-phase HPLC. The yield of **VII** (E) was 60%. The isolated compound was homogeneous by thin-layer and anion-exchange chromatography. $R_f = 0.70$ in the system B; UV (H_2O) λ_{max}, 255 nm (ϵ = 32 600); λ_{min}, 218 nm (ϵ = 17 900). IR (KBr) ν_{max}, 2205 ($-N_3$), 1630 ($-C{=}O_{amid}$) cm^{-1}. ^{31}P NMR (D_2O): δ −21.4 (t, J = 20 Hz, P_β, 1P), −10.7 (d, J = 20 Hz, P_α, 1P), −7.8 (d, J = 20 Hz, P_γ, 1P). ^1H NMR (D_2O): δ 2.37 (t, J = 5.5 Hz, H2′, 2H), 4.03 (m, H3′, H4′, H5′, 4H), 4.10 (d, J = 5.0 Hz, H9, 2H), 6.2−6.4 (m, H1′, H7, H8, 3H), 7.9 (s, H6, 1H).

Synthesis of 5-{*N*-[*N*′-(2-nitro-5-azidobenzoyl)-3-aminopropionyl]aminomethyl}-2′-deoxyuridine-[α-^{32}P]-5′-triphosphate (lithium salt) (VI*b). 5-(Trifluoroacetylaminomethyl)-2′-deoxyuridine was synthesized from derivative **III** (0.1 mmol) by treatment with 1 mmol of ethyl trifluoracetate (120 μL) in methanol (1 mL) in the presence of 1.5 mmol of TEA (207 μL). After stirring for 15 h at 20 °C, the solution was evaporated under reduced pressure. The yellow liquid residue was dissolved in 3 mL of water and applied on a Dowex 50W X2 column (1.5 × 20 cm). 5-(Trifluoroacetylaminomethyl)-2′-deoxyuridine was eluted by water, and fractions were evaporated under reduced pressure. The yield was 94%: R_f = 0.73 in system A.

The obtained deoxyuridine analogue was converted to the 5′-*O*-dimethoxytrityl derivative by conventional procedure (*16*) and subjected to phosphorylation with $POCl_3$ using the known method (*15*). Compound **VIII** was obtained by deprotection by incubation in 0.1 N HCl in methanol (0.5 h) followed by treatment with 10 mL of concentrated aqueous ammonia for 3 h. The solution was evaporated, diluted with 15 mL of water and the product was isolated by ion-exchange chromatography on DEAE-cellulose DE-32 (1 × 20 cm) using a gradient of thriethylammonium bicarbonate (0.00−0.2 M, pH 7.5). The fraction containing **VIII** was evaporated and dried in vacuo. The yield of **VIII** was 53%: R_f = 0.76 in system B; UV (H_2O) λ_{max}, 269 nm (ϵ = 8800).

The overall procedure relies on modification of the method of Walseth and Johnson (*17*) for the enzymatic preparation of [α-^{32}P]nucleoside-5′-triphosphates from 5-(aminomethyl)-2′-deoxyuridine-3′-monophosphate and [γ-^{32}P]ATP.

The [α-^{32}P]photoreactive nucleoside-5′-triphosphate derivative **VI*b** was obtained by succesive acylation of NH_2-group of **X** with trifluoroacetyl-β-alanine *N*-hydroxysuccinimide, and benzoylation of deprotected NH_2-group of the β-alanine with 2-nitro-5-azidobenzoic acid *N*-hydroxysuccinimide as described above for compound **VIa**. The yield of **VI*b** was 83%: R_f = 0.30 in system B.

Studies of the Thermolytic Stability of Azido Group of Photoreactive Nucleoside-5′-triphosphate Derivative VII. The thermolytic stability of azido groups of new compouds under the conditions of PCR was studied using the compound **VII**. A 0.5 mM aqueous solution (100 μL) of photoreagent **VII** was subjected to the thermal regime of the amplification procedure. The dU*TP was precipitated by addition of 2% $LiClO_4$ in acetone. The precipitate was carefully washed with acetone (2 × 1.2 mL). IR (KBr) ν_{max}, 2205 ($-N_3$), 1630 ($-C{=}O_{amid}$) cm^{-1}.

Photochemical Behavior of Photoreactive Amplified DNA. A 10^{-7} M solution (20 μL) of photoreactive amplified [^{32}P]DNA in 0.15 M NaCl, 0.1 mM EDTA, and 0.02 M Na_2HPO_4 (pH 9) was pipetted into a 96-well polystyrene plate at 4 °C. Irradiation was carried out by exposing the plate for 18 min to filtered light (300−365 nm) emitted by a high-pressure mercury lamp with an intensity 10^{15} quanta × cm^{-2} × s^{-1}. The irradiated mixture was subjected to electrophoresis in 10% denaturing PAAG and vizualized by radioautography. Quantitative treatment of radioautographs was carried out using Ultrascan laser densitometer (LKB).

RESULTS AND CONCLUSIONS

Synthesis and Characterization of Photoanalogues dU*TP. Synthesis of new dUTP derivatives

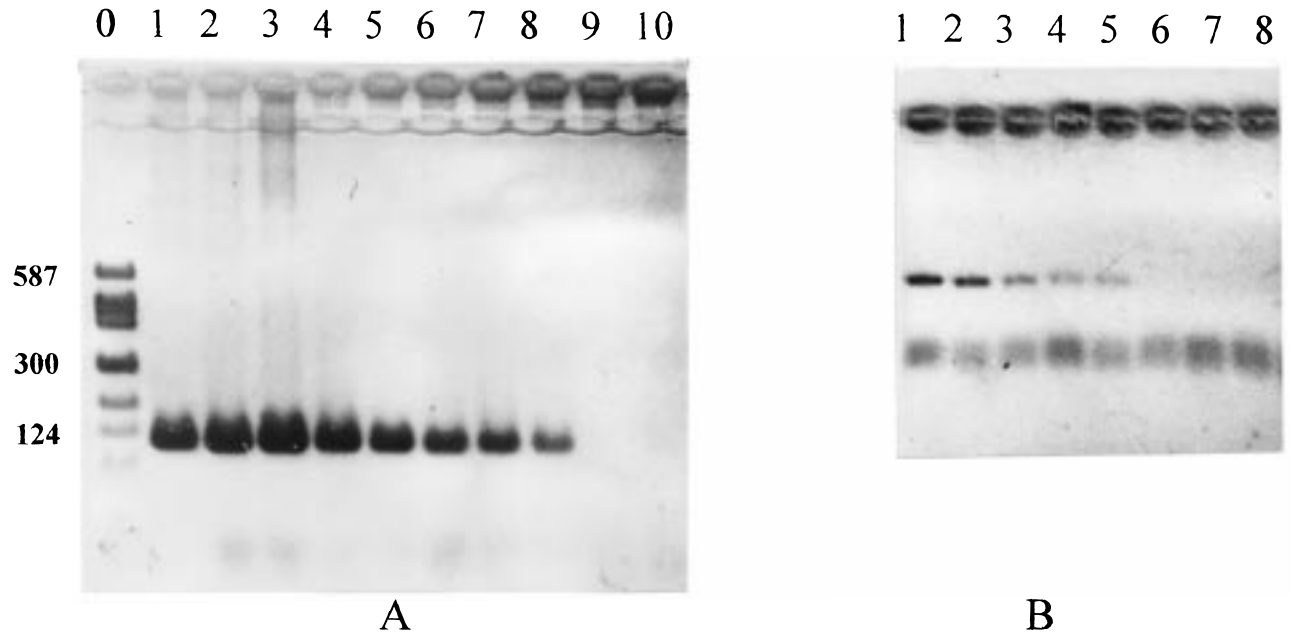


Figure 1. The product of the PCR in the presence of different amount of dU*TP analogue **VIa** (A); analogue **VIb** (B). (A) Lane 1, dTTP only; 2, 10% **VIa**; 3, 20% **VIa**; 4, 50% **VIa**; 5. 60% **VIa**; 6, 70% **VI**a; 7, 80% **VIa**; 8, 85% **VIa**; 9, 90% **VIa**; 10, 100% **VIa**. (B) Lane 1, dTTP only; 2, 10% **VIb**; 3, 20% **VI**; 4, 50% **VIb**; 5, 60% **VIb**; 6, 70% **VIb**; 7, 80% **VIb**; 8, 100% **VIb**; 4 μL from a 50 μL PCR product was analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Lane 0 (A), the molecular size marker is pGem-1 (HAE III).

(dU*TP), carrying photoreactive groups, which are highly reactive toward both DNA and proteins were carried out as shown in Schemes 1 and 2.

It is known that the introduction of CH═CH−CH_2−NH_2 spacer to 5-C of 2′-deoxyuridine is accompanied by the formation of isomers with the trans propenyl configuration being the major product (*6*, *18*). We have isolated three isomers of compound **VII** by reversed-phase HPLC. We have proposed that they are E and Z isomers and 5-[3-(4-azido-2,3,5,6-tetrafluorobenzamido)propenyl-2]-2′-deoxyuridine-5′-triphosphate—the product of arylation of allylamine in C2 position, on the basis of ref *18*.

Compounds **I**−**IV** were obtained using the known method (*14*). Reaction of **IV** with $POCl_3$ resulted in the formation of a mixed anhydride, which produced nucleoside-5′-triphosphate **V** under interaction with bis(tri-*n*-butylammonium)pyrophosphate and the following treatment of the reaction mixture with concentrated aqueous ammonia, permitted to remove CF_3CO residue quantitatively off the β-alanyl NH_2-group. Photoreactive derivatives **VIa**,**b** were obtained by benzoylation of aliphatic amino group of **V** with *N*-hydroxysuccinimide esters of 4-azido-2,3,5,6-tetrafluorobenzoic or 2-nitro-5-azidobenzoic acids. The use of the triethylammonium salt of **V** permitted to carry out the reaction in DMF in the presence of triethylamine, thus opening the possibility to achieve higher yields (near 90%) with lower excesses of *N*-hydroxysuccinimide esters.

The structure of photoreactive dU*TP derivatives **VIa**,**b** and **VII** (E) was verified by ^{1}H NMR. The data of anion-exchange chromatography and ^{31}P NMR spectroscopy [the presence of the resonance at δ −21.4 ppm (t, J = 20 Hz, P_β, 1P), −10.7 (d, J = 20 Hz, P_α, 1P), −7.8 (d, J = 20 Hz, P_γ, 1P), for one example, the compound **VII**] proved the presence of the 5′-triphosphate moieties. IR spectra of the compounds obtained revealed the presence of the band at 2205 cm^{-1} (KBr) characteristic of the azido group.

Each photoreactive dU*TP analogue was examined to answer the following questions. Is the dU*TP derivative a substrate for DNA polymerases? Is the newly synthesized DNA containing photoreactive groups a template for the following DNA replication? Is it able to efficiently cross-link with template DNA under irradiation?

Consequently, we have carried out the polymerase chain reaction (PCR) using DNA polymerase *Thermus aquaticus* assays and offering our photoreactive nucleoside-5′-triphosphates as substrates both in the presence and in the absence of dTTP. The amplification of DNA was performed using a pair of oligodeoxyribonucleotide primers which flank a 110 bp region in the gene of the envelope protein E of the tick-borne encephalitis virus. Optimal conditions of PCR for this system with natural nucleoside-5′-triphosphates were elucidated earlier (*19*).

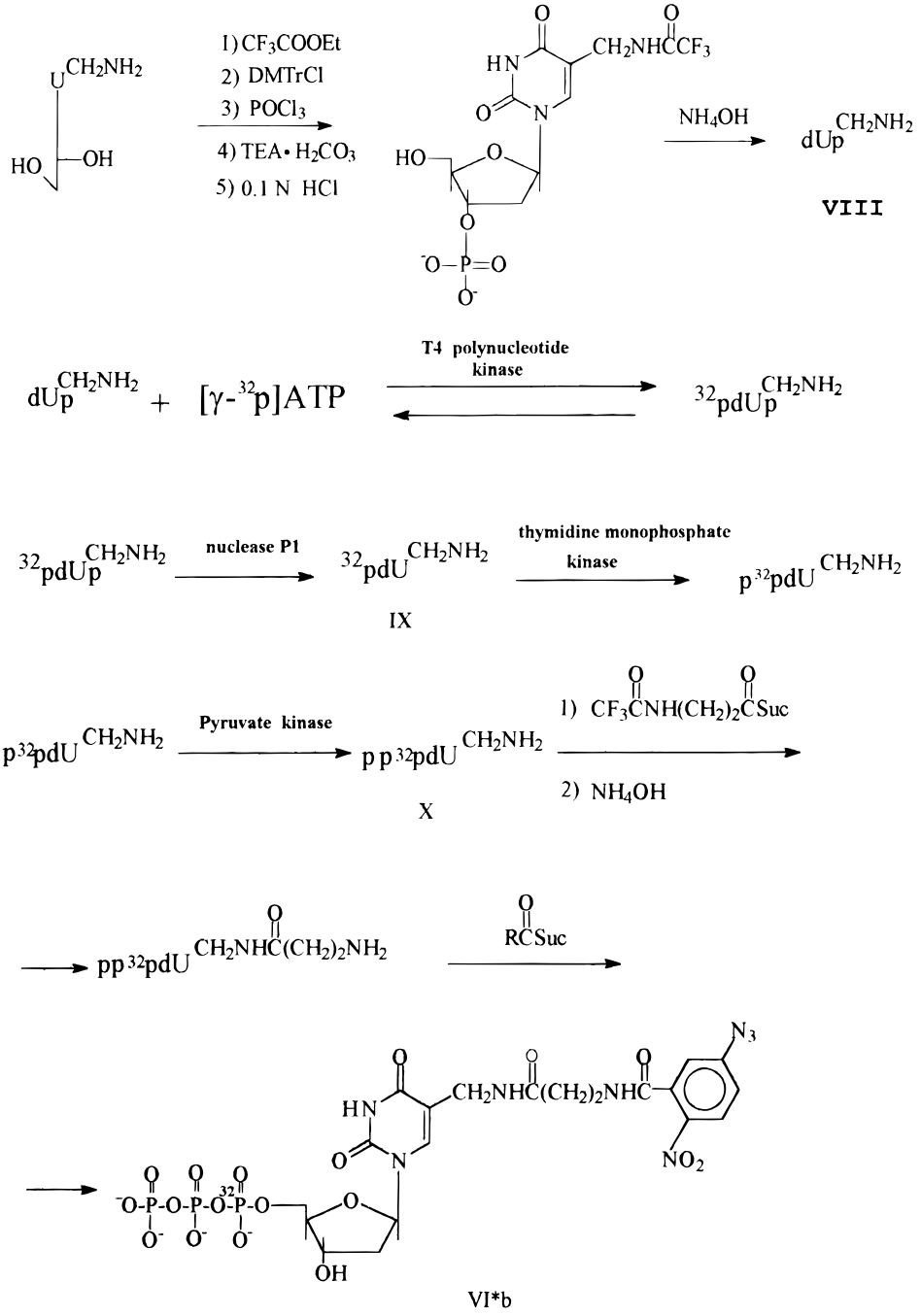
Since azides are known to undergo thermolytic decomposition, the stability of photoreactive nucleoside-5′-triphosphates was preliminary investigated. The thermolytic stability of azido groups of new compounds under the conditions of PCR was studied using the compound **VII**. A 0.5 mM aqueous solution (50 μL) of photoreagent **VII** was subjected to the terminal regime of the amplification procedure. The dU*TP was precipitated by addition of 2% $LiClO_4$ in acetone. The presence of an azido group in the product was proved by IR spectroscopy (the presence of distinct band at 2205 cm^{-1}). The ratio of intensities ν_{2205}/ν_{1630} did not change after treatment in the thermal PCR regime.

When the reaction mixture for PCR with photoanalogues **VIa**,**b** were used in the absence of dTTP, no traces of the full-length primer extension product was observed (Figure 1). The amplified DNA appeared only in the case of simultaneous presence of certain amount of both photoreactive dU*TP and dTTP.

To establish if dU*TP can be used by the DNA polymerase as the substrate, the incorporation of [α-^{32}P]-dU*TP analogue **VIb** in the amplified product was estimated.

Synthesis of the photoreactive [α-^{32}P]dU*TP was fulfilled according to the Scheme 3. Deoxyuridine analogue **III** was converted to 5′-*O*-dimethoxytrityl derivative by

Scheme 3



a conventional procedure and subjected to phosphorylation with $POCl_3$ with subsequent hydrolysis by triethylammonium bicarbonate and deprotection of 5′-OH group by incubation in 0.1 N HCl. The 2′-deoxyuridine-3′-monophosphate analogue **VIII** obtained was enzymatically converted to [5′-^{32}P]2′- deoxyuridine-3′,5′-diphosphate using [γ-^{32}P]ATP and T4 polynucleotide kinase. Then 3′-phosphate was removed by nuclease P1. [5′-^{32}P]-dU*MP (**IX**) was further phosphorylated to dU*DP with nonradioactive ATP using thymidine monophosphate kinase from *E. coli* MRE-600 and to dU*TP (**X**) with phosphoenolpyruvate using pyruvate kinase. Interestingly, despite its high specificity, dTMP kinase turned out to be able to catalyze phosphorylation of **IX**, albeit at lower efficiency as compared with dTMP. The same results were obtained for the analogue with protecting trifluoroacetyl residue on aliphatic amino group.

The [α-^{32}P]photoreactive nucleoside-5′-triphosphate derivative **VI*b** was obtained by succesive acylation of NH_2-group of the compound **X** with trifluoroacetyl-β-alanine *N*-hydroxysuccinimide, removal of trifluoroacetyl residue, and benzoylation of deprotected NH_2-group of the β-alanine with *N*-hydroxysuccinimide ester of 2-nitro-5-azidobenzoic acid as described above for **VIa**.

The product was separated from reaction mixture by ion-exchange and reversed-phase chromatography and analyzed by thin-layer chromatography (TLC). The radiolabeled dU*TP (**VI*b**) had $R_f = 0.3$ on DC-Alufolien Kieselgel TLC plates in the system (B), which is identical with that for nonradioactive photoreactive dU*TP derivative **VIb**.

When the polymerase chain reaction was carried out using the mixture of 50% of photoreactive [α-^{32}P]2′-deoxyuridine-5′-triphosphate derivative **VI*b** and the

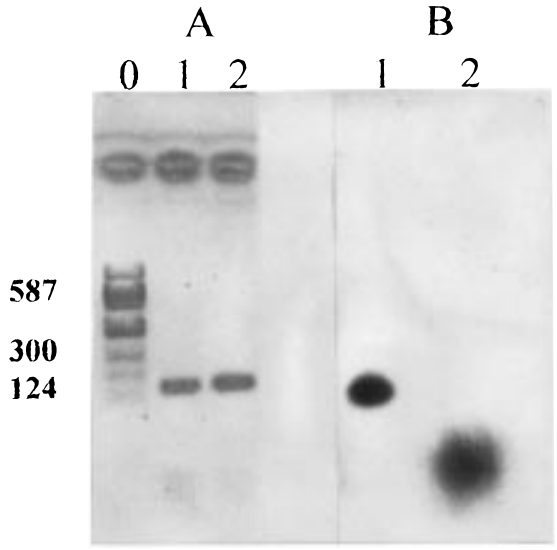


Figure 2. The product of the PCR carried out with 100% dTTP and ^{32}P-foward primer (lane 1); with 50% compound **VI*b** and 50% dTTP (lane 2); lane 0, the molecular size marker as Figure 1. A total of 4 μL from a 50 μL PCR product was analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining (A), autoradiography (B).

dTTP (50%), the formation of full-length amplificate band was observed, as revealed by ethidium bromide staining of the gel (Figure 2A). However, the respective band did not contain any radioactivity (Figure 2B). The radioactive label was observed only in shorter fragments. This indicates that incorporation of the analogue does not permit further elongation. On the contrary, it was found that **VII** (E) replaced dTTP during synthesis of DNA by PCR as revealed by the formation of the full-length amplificate in the presence of this dU*TP analogue without any dilution with natural dTTP (Figure 3). In the same time, we did not obtain full-length amplificate using compounds **VII** (Z) and 5-[3-(4-azido-2,3,5,6-tetrafluorobenzamido)propenyl-2]-2′-deoxyuridine-5′-triphosphate.

The PCR mixture was subjected to electrophoresis in 10% PAAG. The amplified DNA was extracted from the gel by electroelution and inspected by UV and IR spectroscopy. The UV absorption spectra of the product (2×10^{-4} M, H_2O) showed λ_{max} 260 nm characteristic of the oligonucleotide component. IR spectroscopy (2×10^{-4} M, H_2O, 0.02 mm CaF_2 cuvette) proved that analogue **VII** (E) was included into the DNA by PCR (the presence of the characteristic band of the azido group at 2100 cm $^{-1}$).

The photolytic behavior of photoreactive amplified DNA was investigated. The solution of amplified [^{32}P]-DNA (10^{-7} M) in 0.15 M NaCl and 0.02 M Na_2HPO_4 (pH 9) was subjected to photolysis for 18 min and analyzed by denaturing gel electrophoresis. The irradiation time was chosen to be 10-fold greater than the half-life of photolysis. The kinetics of photolysis was studied by recording the IR spectra (2000−3000 cm^{-1}) of the samples irradiated for definite time intervals. As shown in Figure 4, irradiation of the full-length amplificate resulted in the appearance of the cross-linked amplification product (peak 2).

To prove additionally the ability of **VII** (E) to serve a substrate of DNA polymerases, we studied its incorporation into the system: 3′-GGGTCAGTGCTGCAACATTTT-GCTGC... (as a template) and [^{32}P]CCCAGTCACGACGT (as a primer).

It can be seen in Figure 5 that the compond **VII** (E) is incorporated into the DNA chain by the Klenow fragment

Figure 3. The product of the PCR carried out with 100% compound **VII** (E) (lane 1); with 100% dTTP (lane 2). A total of 4 μL from a 50 μL PCR product was analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

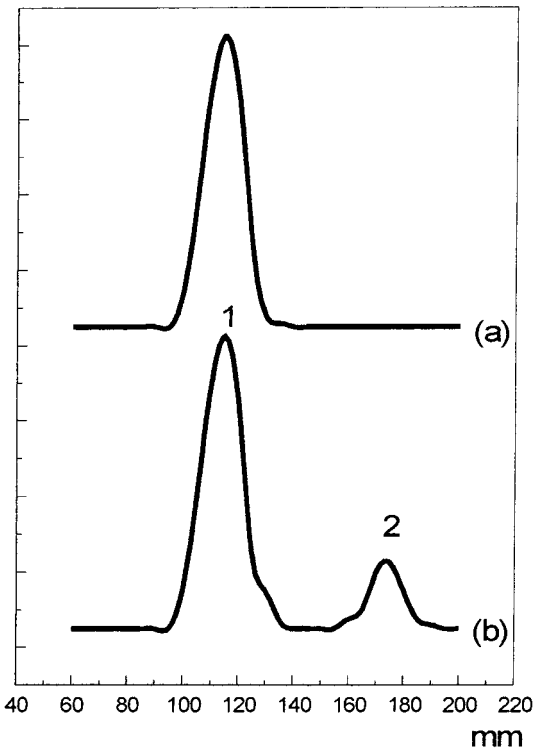


Figure 4. Densitogramms of the gel-radioautographs for the product of the PCR were carried out with 100% compound **VII** and ^{32}P-forward primer. Nonirradiated amplified DNA (a). Irradiated product (b).

of DNA polymerase I (lanes 4−9) and *Taq* DNA polymerase (lanes 13−15). In the control assays, tetradecanucleotide was extended by one residue in the presence of dTTP (lanes 2 and 11) and by three residues in the presence of dTTP and dGTP (lanes 3 and 12) giving

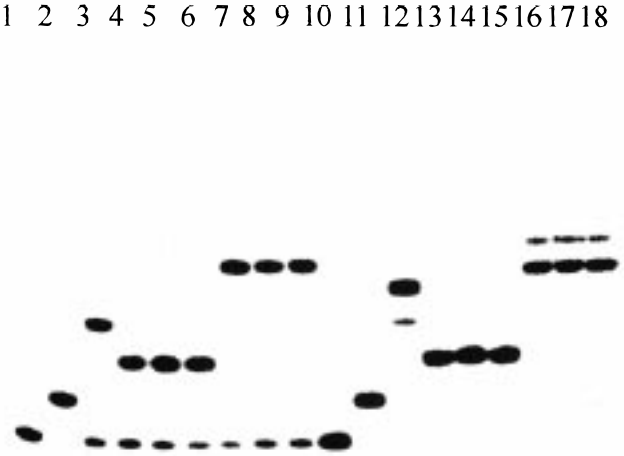


Figure 5. Primer extension catalyzed by Klenow fragment of DNA polymerase I (lanes 1−9) and *Taq* DNA polymerase (lanes 10−18). lanes 1 and 10, enzyme + primer-template 3′GGGTCAGTGCTGCAACATTTTGCTGC (the M13mp10 phage DNA) [^{32}p]CCCAGTCACGACGT (the primer); lanes 2 and 11, as in lanes 1 and 10 + 2 μM dTTP; lanes 3 and 12, as in lanes 2 and 11 + 2 μM dGTP+; lanes 4−6 and 13−15, as in lane 1 + 0.5 μM, 2 μM, and 8 μM compound **VII** (E); lanes 7−9 and 16−18, as in lanes 4−6 and 13−15 + 2 μM dGTP. The reaction products were separated by electrophoresis in the denaturing 20% PAAG.

15-mer and 17-mer, respectivelly. Additionally, there is an 18-mer band most probably due to misincorporation of dGMP residue with formation of mismatched T:G pair, which is most stable among noncanonic pairs (*20*). In the case of the Klenow fragment of *E. coli* DNA polymerase I, displaying 3′-5′ exonuclease activity, which is absent in *Taq* DNA polymerase, low level of a mismatched incomplete nucleotide at the 3′-end was observed. This has been noticed many times before in the systems with a reduced set of substrates (*21*, *22*). Incorporation of one residue of **VII** (E) into a nascent DNA chain would result in formation of a hydrophobic 15-mer of lower electrophoretic mobility (lanes 4−6) as compared with its natural counterpart (lane 2). After addition of dGTP to the assay containing **VII,** one can see the elongation of 15-mer band by two nucleotides (17-mer band) according to the template context (lanes 7−9). The position of 17-mer band corresponds to the mobility of oligonucleotide with two hydrophobic residues. So, we can state that Klenow fragment of *E. coli* DNA polymerase I and *Taq* DNA polymerase are able to incorporate the modified deoxyuridine-5′-phosphate residue with large hydrophobic photoreactive substituents at the 5-position into the 3′-end of the DNA chain keeping the ability of further elongation. Thus, it was shown that the use of modified substrate did not lead to the loss of the specificity of template synthesis.

The unique property of dU*TP analogue **VII** (E) made it possible to use it for direct incorporation in the course of elongation of DNA probes at the template DNA in chromosomes and in interphase chromatin with subsequent cross-linking of the elongated probes with matrixed DNA. This ability might provide a possibility of localization of short probes. We have used this approach for localization of nucleotide sequences on human metaphase chromosomes and interphase nuclei. These results presented in a separate communication (*23*).

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