Synthesis of Mono- and Dinucleotide Photoaffinity Probes of Ribonucleic Acid Polymerase†

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ABSTRACT: The abortive initiation reaction of RNA polymerase has been used to prepare adenylyl-(3′–5′)-uridine 5′-phosphate (pApU) in 74% yield from AMP and UTP. The reactive intermediate *p*-azidophenyl phosphorimidazolidate has been prepared by starting from *p*-nitrophenyl phosphate. Reaction of this compound with the terminal phosphates of adenosine 5′-phosphate and adenylyl-(3′–5′)-uridine 5′-phosphate gives the corresponding β-substituted 5′-diphosphates. These products are incorporated into the 5′ (leading) end of RNA by RNA polymerase (*Escherichia coli*) and can be photoactivated at a specific stage of RNA elongation. The dinucleotide photoaffinity label β-(4-azidophenyl) adenylyl-(3′–5′)-uridine 5′-diphosphate stimulates RNA synthesis more strongly than adenylyl-(3′–5′)-uridine.

RNA polymerase (EC 2.7.7.6) is a multisubunit enzyme which catalyzes the synthesis of RNA from nucleoside triphosphates, using DNA as a template. Synthesis proceeds in the 5′ to 3′ direction, and the residue at the 5′ end may be as small as a nucleoside or as large as NAD or FAD (Armstrong & Eckstein, 1976; Yarbrough et al., 1979; Malygin & Shemyakin, 1979). This property suggested to us that chemical or physical probes, such as photoaffinity labels, could be incorporated at the leading (5′) end of the nascent RNA molecule by RNA polymerase. These probes could then provide information about their surroundings as the nucleotide chain was elongated. In particular, photoaffinity probes could lead to identification of those enzyme subunits which make up the path of nascent RNA across the surface of RNA polymerase.

We have synthesized mono- and dinucleotides containing photoreactive aryl azides coupled to the 5′-phosphate. Aryl azide photoaffinity probes were used because they are not chemically reactive until activated by photolysis, and the nitrenes formed by photolysis react rather indiscriminately with nearby molecules (Knowles, 1971; Bayley & Knowles, 1977). Dinucleotides have been shown to be much more efficient at initiating RNA synthesis than mononucleotides (Downey & So, 1970). We have found that the "abortive initiation" reaction of RNA polymerase provides a convenient, high-yield route to the synthesis of dinucleotides. The term abortive initiation describes the catalytic production of dinucleotides (or other oligonucleotides) by RNA polymerase in the presence of a DNA template (Johnston & McClure, 1976). The dinucleotide pApU[1] was made in this way from AMP and UTP, using poly[d(A-T)] as the template. The dinucleotide photoaffinity probe β-(4-azidophenyl) adenylyl-(3′–5′)-uridine 5′-diphosphate, denoted N_3PhppApU, was then synthesized by reaction of pApU with *p*-azidophenyl phosphorimidazolidate. A mononucleotide photoaffinity probe, β-(4-azidophenyl) adenosine 5′-diphosphate (N_3PhppA), was similarly prepared. Initiation of RNA synthesis using the dinucleotide photoaffinity label occurred efficiently at much lower concentrations than initiation with the mononucleotide affinity label, as described below.

Experimental Procedures

Materials

All reagents and solvents were reagent grade and used without further purification unless noted otherwise. Deionized and distilled water was used throughout. *p*-Nitrophenyl phosphate and *N*,*N*′-carbonyldiimidazole were from Aldrich. Bacterial alkaline phosphatase, snake venom phosphodiesterase, apyrase, and Tris were from Sigma. Tris was recrystallized from methanol prior to use. Mononucleotides were from Sigma or P-L Biochemicals, Inc. Poly[d(A-T)] was from P-L Biochemicals, Inc. α- and γ-^{32}P-labeled nucleoside triphosphates were from Amersham. Tritiated UTP and Aquasol scintillation cocktail were from New England Nuclear. Silica gel 60 F_{254} and PEI–cellulose F thin-layer chromatography plates were from Merck. Sephadex A-25 was from Pharmacia, and AG-50 resin was from Bio-Rad. Kodak type XR X-ray film was used for autoradiograms. Type HA nitrocellulose filters and Pellicon type PT membranes, M_n 10 000, were purchased from Millipore.

Methods

Thin-Layer Chromatography. Both PEI–cellulose and silical gel plates were used. The former were faster, but R_f's were highly dependent upon the ionic strength of the applied sample solution. PEI–cellulose plates were washed in 1 M NaCl, rinsed with H_2O, and dried before use. Silica gel plates were used when it was necessary to compare samples with different ionic strengths, especially enzymatic digests.

The solvent systems used were as follows: (solvent A) 2-propanol/concentrated NH_4OH/H_2O 6/3/1 (v/v/v) (Block et al., 1955); (solvent B) 1 M acetic acid (1 cm of plate) and 0.3 M LiCl (remaining 4 cm) (Randerath, 1966); (solvent C) 1 M formic acid and 0.25 M LiCl (Randerath, 1966); (solvent D) 0.7 M $MgCl_2$.

Aromatic compounds were visualized by fluorescence quenching. Radioactive spots were located by scraping the chromatography medium from the TLC plate into scintillation

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[1] Abbreviations used: DMF, *N*,*N*-dimethylformamide; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; N_3Php, *p*-azidophenyl phosphate; pApU, adenylyl-(3′–5′)-uridine 5′-phosphate; N_3PhppApU, β-(4-azidophenyl) adenylyl-(3′–5′)-uridine 5′-diphosphate; N_3PhppA, β-(4-azidophenyl) adenosine 5′-diphosphate; PEI, poly(ethylenimine); M_n, nominal molecular weight limit.

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vials, suspending the particles in the scintillation cocktail (gelatinous phase), and counting.

Buffers. The buffers used were as follows: (E) 10 mM NaCl and 10 mM Tris-HCl, pH 7.9; (F) 80 mM Tris-HCl, pH 7.9, 5 mM β-mercaptoethanol, 50% (v/v) glycerol, and 0.1 mM EDTA.

Nucleotides and Template. Nucleotides were obtained from Sigma and P-L Biochemicals, Inc. ATP and UTP were each purified by two passages through a Sephadex A-25 column in the formate form. A linear gradient of 0.01–0.75 M ammonium formate, pH 6.5–7.0, was used each time. Excess salt and solvent were removed under reduced pressure at room temperature. Concentrations of neutral aqueous solutions were determined by measuring absorbances at 260 nm (Dawson et al., 1969). Solutions were stored at −20 °C. The purity of radiolabeled compounds was determined by autoradiography of thin-layer chromatograms on PEI–cellulose plates developed by using solvents B and C. No purification of the ^{32}P-labeled compounds was necessary.

Poly[d(A-T)] was dialyzed against buffer E for 2–3 days. Concentrations were determined by measuring absorbance at 260 nm and using the technical data supplied by P-L Biochemicals, Inc. Poly[d(A-T)] solutions were stored at −20 °C.

RNA Polymerase. RNA polymerase was isolated from *E. coli* MRE 600 cells (purchased from Grain Processing Corp.) by using the method of Burgess & Jendrisak (1975) with recent modifications (Lowe et al., 1979). $NaDodSO_4$–urea polyacrylamide gel electrophoresis showed only the subunits α, β, β′, and σ normally associated with the enzyme. The enzyme was dialyzed into buffer F and was stored at −79 °C in 50-μL aliquots. Protein concentrations were determined by using a modification of the Bradford procedure (Bradford, 1976; Sedmak & Grossberg, 1977), with bovine serum albumin as a standard. A stock solution containing 6.48 μg/μL RNA polymerase in buffer F was used throughout the study unless noted otherwise.

Synthesis of Photoaffinity Labels. N,N-Dimethylformamide (DMF) was dried by vacuum distillation (Hilgetag & Martini, 1972) and stored in a desiccator. Columns were eluted with linear gradients throughout. All manipulations of azide-containing compounds were done in reduced light (using a 7.5-W bulb) or total darkness, unless noted otherwise. NMR spectra (360 MHz) were taken by Dr. R. Anderson on the Nicolet Instrument at the UCD magnetic resonance facility. All NMR spectra were taken in D_2O with the HDO peak assigned a chemical shift of 4.8 ppm.

p-Azidophenyl Phosphorimidazolidate. Disodium *p*-nitrophenyl phosphate hexahydrate (101 mg, 0.27 mmol) was dissolved in 25 mL of H_2O and the pH was adjusted to 6.5 by addition of 3.5 μL of 6 M HCl. Ten percent Pd/C catalyst (29.5 mg) was added to the solution, and the solution was placed under 1 atm of H_2. Reduction was allowed to proceed at room temperature until hydrogen consumption ceased (1 h). The catalyst was removed by filtration, and the filtrate was taken to dryness under reduced pressure. The product (*p*-aminophenyl phosphate) gave a yellow spot with fluorescamine (Udenfriend et al., 1972) and had R_f 0.68 on PEI–cellulose, using solvent B; with the same TLC system, *p*-nitrophenyl phosphate had R_f 0.52. The 100-MHz NMR spectrum of *p*-aminophenyl phosphate showed an AB pattern with peaks at 6.8, 6.9, 7.0, and 7.1 ppm.

The azide was prepared via the diazonium salt as described by Smith & Knowles (1974). After the reaction was complete, the solution was adjusted to pH 5.5 by the addition of 60 μL of 2.0 M NaOH. The azide was purified on a Sephadex A-25 column, 1 × 23 cm, acetate form, using a gradient of 0.01–0.75 M ammonium acetate, pH 6.0–6.7. The effluent absorbance was monitored at 310 nm in order to detect the product without significantly photolyzing it. The product eluted between 0.25 and 0.32 M ammonium acetate. Excess salt and solvent were removed under reduced pressure. On a PEI–cellulose TLC plate developed in solvent B, the product gave one fluorescence quenching spot with R_f 0.46. Exposure to 254-nm radiation from a Mineralight UV-11 lamp caused the spot on the TLC plate to turn dark brown. This photoinduced browning was used to identify the azide moiety in later syntheses. The 100-MHz NMR spectrum of the product had an AB pattern with peaks at 7.1, 7.2, 7.3, and 7.4 ppm. The UV absorption spectrum of the product in H_2O had an absorbance maximum at 250 nm with a slight shoulder at 280 nm. The IR spectrum of the product showed a doublet at 2100 and 2130 cm^{-1} which is characteristic of azides (Dyer, 1965). UV photolysis of the KBr pellet containing the azide caused a reduction in the intensity of these IR absorbances. The yield of azide was 75% (determined by using $\epsilon_{260} = 1.03 \times 10^4$ M^{-1} cm^{-1} (Maassen & Möller, 1974)). Aqueous solutions of the azide were stable for at least a year if stored in the dark at −79 °C.

Methyl(tri-*n*-octyl)ammonium hydroxide was prepared as described by Michelson (1963) and standardized by titration against potassium hydrogen phthalate. The ammonium salt of *p*-azidophenyl phosphate was converted to the acid form by passage through an AG-50 column, H^+ form. The mono[methyl(tri-*n*-octyl)ammonium] salt of *p*-azidophenyl phosphate was prepared (Barker et al., 1972) and taken to dryness 3 times from dry DMF. The glassy solid (235 μmol) was dissolved in 1.38 mL of dry DMF and 0.191 g (1.18 mmol) of *N,N*′-carbonyldiimidazole was added. The 25-mL flask containing the reaction and a magnetic stir "flea" was stoppered and placed in a light-tight desiccator on a stir plate. After 24 h, the reaction was checked by thin-layer chromatography on PEI–cellulose by using solvent B. The *p*-azidophenyl phosphate had been completely converted to the *p*-azidophenyl phosphorimidazolidate, which had R_f 0.86. Anhydrous methanol (48 μL, 1.18 mmol) was added to quench unreacted *N,N*′-carbonyldiimidazole, and the reaction was allowed to stand 30 min at room temperature before the solution was added to a DMF solution containing a nucleotide.

Adenylyl-(3′–5′)-uridine 5′-Phosphate (pApU). The abortive initiation reaction catalyzed by RNA polymerase was used to synthesize pApU (Johnston & McClure, 1976). The reaction mixture contained the following in a volume of 10 mL: 20 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 5.3 mM AMP, 4.5 mM UTP (10 μCi/mL of [^{3}H]UTP), poly[d(A-T)] (20 μM phosphate), 2 mg of *E. coli* RNA polymerase, 2.8% (v/v) glycerol, and 1 unit of inorganic pyrophosphatase. The reaction was incubated at 37 °C for 3 days and was monitored on silica gel TLC plates by using solvent system A (product R_f 0.25). Four units of apyrase was added to the reaction mixture (to degrade UTP to UMP), and the mixture was incubated 3–4 h longer. Enzymes and template were removed by filtration through a Pellicon type PT membrane which retains proteins with $M_r > 10^4$. The product was purified on a 1 × 38 cm Sephadex A-25 column, in the acetate form. A 900-mL gradient of 0.01–1.0 M ammonium acetate, pH 5, was used. The product, identified by absorbance at 260 nm and tritium radioactivity, eluted between 0.52 and 0.6 M ammonium acetate. Solvent and salt were removed under reduced pressure at room temperature.

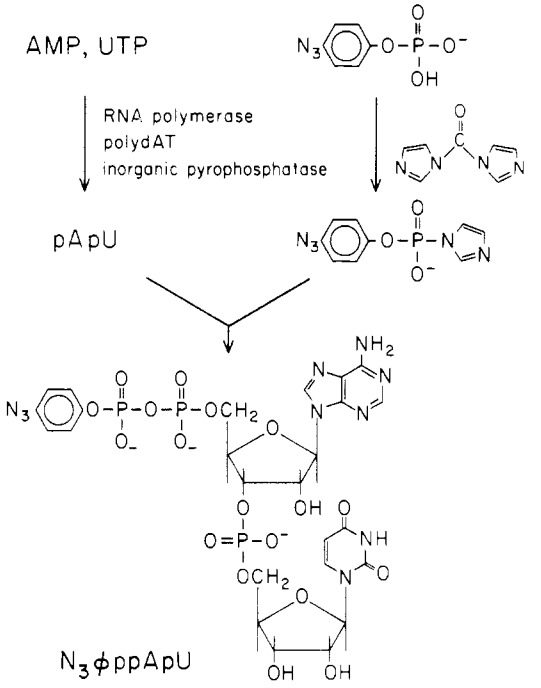

FIGURE 1: Synthesis of the dinucleotide photoaffinity probe N_3PhppApU. In this and subsequent figures, ϕ = Ph = C_6H_4.

The product was identified by 360-MHz proton NMR spectroscopy and by enzymatic digests followed by analysis on silica gel thin-layer plates using solvent system A. The proton NMR spectrum of the product contained resonances corresponding to adenine and uridine in 1:1 stoichiometry. Enzymatic digests of the product were done at 37 °C for 30 min in the buffers given for the appropriate enzyme in the Worthington Enzyme Manual (Decker, 1977). Enzymatic digests and the appropriate standard compounds (ApU, AMP, UMP, A, U) were all applied to the same TLC plate and run in parallel; the compounds were located by fluorescence quenching and tritium radioactivity. A bacterial alkaline phosphatase digest of the product gave adenylyl-(3′–5)-uridine (ApU). AMP and UMP were released in a snake venom phosphodiesterase digest of the product. An incubation of the product with both snake venom phosphodiesterase and alkaline phosphatase gave both adenosine and uridine. Tritium activity was monitored on all the chromatograms; in each of the digests more than 90% of the radioactivity was associated with the expected uridine-containing compound.

The yield of the product adenylyl-(3′–5′)-uridine 5′-phosphate (pApU) was determined to be 33.4 μmol (74%) by using the absorbance of a solution in 0.1 M HCl at 258 nm and assuming that the molar extinction coefficient was equal to the extinction coefficient for ApU (P-L Biochemicals, 1980).

β-(4-Azidophenyl) Adenylyl-(3′–5′)-uridine 5′-Diphosphate (N₃PhppApU). *p*-Azidophenyl phosphate was coupled to pApU by a modification of the methodology developed by Kozarich et al. (1973) for the synthesis of nucleoside diphosphates from nucleoside monophosphates; the procedure is outlined in Figure 1. The ammonium salt of tritium-labeled pApU (33 μmol) was converted to the acid form by elution through an AG-50 (H^+) column and then was converted to the bis[methyl(tri-n-octyl)ammonium] salt as described above for *p*-azidophenyl phosphate. Solvent was removed under reduced pressure. An attempt was made to dissolve the glassy solid in 3 mL of dry DMF, but only after 0.5 mL of ethanol, 1 mL of methanol, and 1 mL of water were added did the solid dissolve. After this solution was evaporated to dryness, the solid dissolved easily in dry DMF and was taken to dryness

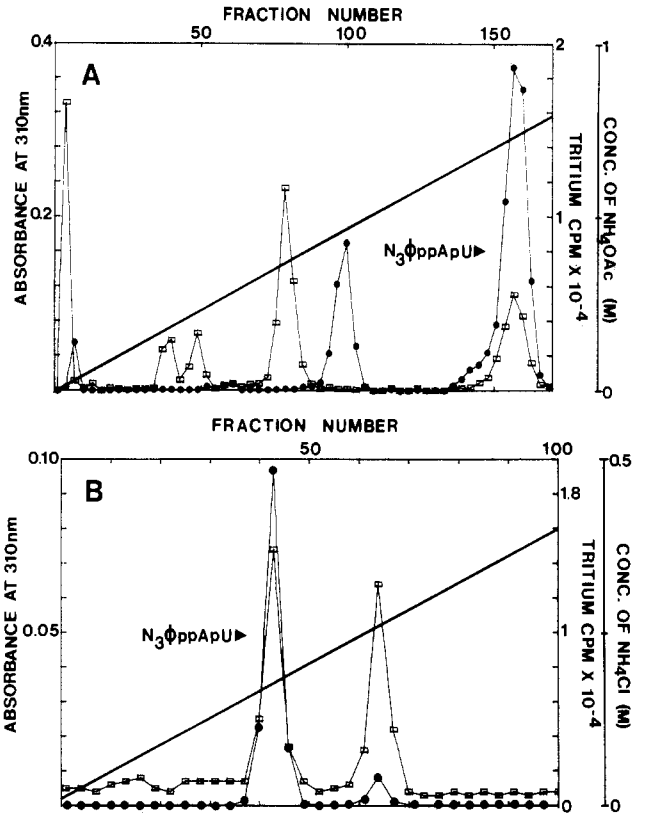

FIGURE 2: Chromatography column elution profiles for purification of N_3PhppApU. Both columns were run at room temperature in the dark. Absorbance at 310 nm (□) and tritium radioactivity (●) were measured for every third fraction. (A) Chromatography of the reaction mixture used to prepare N_3PhppApU on a 1 × 27 cm Sephadex A-25 (acetate) column. As indicated by the heavy line, a gradient of 0.01–1.0 M NH_4OAc, pH 5, was used. Nothing eluted after 0.8 M NH_4OAc. The impurity $N_3PhppPhN_3$ coeluted with N_3PhppApU in the last peak. (B) Impure N_3PhppApU from the column in (A) was eluted from a 1 × 27 cm Sephadex A-25 (chloride) column with a 0.01–1.0 M NH_4Cl gradient (heavy line), at pH 2.5. The first peak contained pure N_3PhppApU. Nothing eluted after 0.30 M NH_4Cl.

out of DMF 2 more times. The bis[methyl(tri-*n*-octyl)ammonium]salt of pApU was taken up in 200 μL of dry DMF; 200 μL more of DMF and 110 μL of a DMF solution containing 165 μmol of *p*-azidophenyl phosphorimidazolidate were added with stirring. The reaction was monitored by TLC on PEI–cellulose using solvent system C; the product had R_f 0.40. After 39 h, 61% of the nucleotide had been converted to product; the reaction was stopped by removal of the solvent under reduced pressure. The residue was taken up in water and methanol and the resulting homogeneous solution was applied to a 1 × 27 cm Sephadex A-25 column, acetate form. The column was eluted with a gradient of 0.01–1.0 M ammonium acetate, pH 5, total volume 1 L. The radioactivity and absorbance at 310 nm were measured for every third fraction; the column trace is shown in Figure 2A. The product coeluted with an azide-containing impurity between 0.72 and 0.78 M ammonium acetate. Salt and solvent were removed under reduced pressure. On silica gel TLC with solvent A the product had R_f 0.51 and the photosensitive but nonnucleotide impurity had R_f 0.71. The yield of product was determined to be 18.2 μmol (55%), based on tritium radioactivity. Half of the impure product was applied to a 1 × 27 cm A-25 column, Cl^- form, with a gradient of 0.01–1.0 M NH_4Cl, pH 2.5, total volume 1 L. The effluent was monitored as described; as shown in Figure 2B, pure product eluted between 0.15 and 0.20 M salt. Water was removed under reduced pressure. Salt was removed by elution of the sample from a

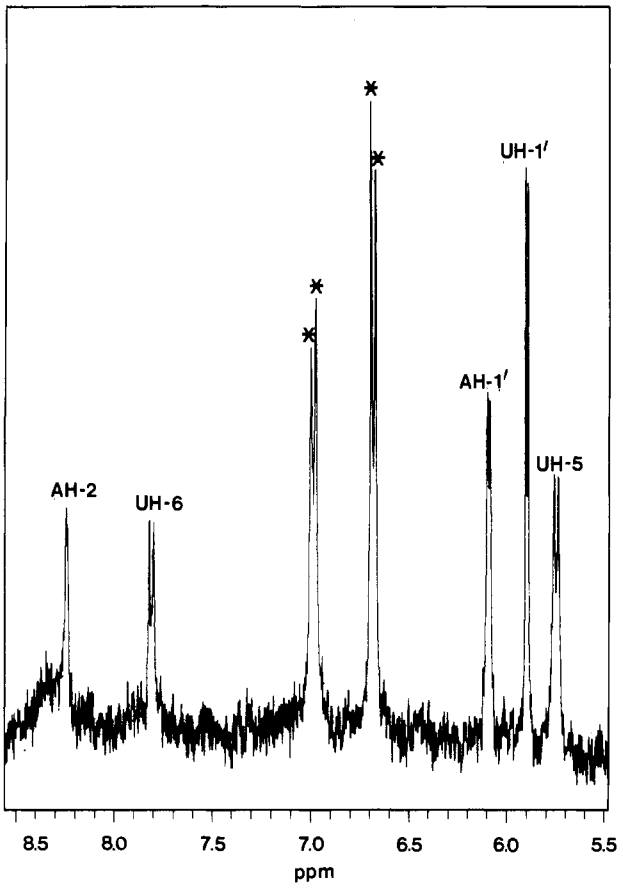

FIGURE 3: Aromatic region of the 360-MHz NMR spectrum of N_3PhppApU in D_2O at a pH meter reading of 7.0. The proton resonances corresponding to the *p*-azidophenyl phosphate moiety are indicated by asterisks. The resonances corresponding to the protons on the 2 and 1′ carbons of adenosine are designated AH-2 and AH-1′; those corresponding to protons on the 5, 6, and 1′ positions of uridine are designated UH-5, UH-6, and UH-1′, respectively. Assignments were made according to Ts'o et al. (1969).

2.5 × 50 cm Sephadex A-25 column, acetate form, with a 1.6-L gradient of 0.1–2.0 M ammonium acetate, pH 7.0. The product eluted between 1.24 and 1.48 M salt. Excess salt and water were removed under reduced pressure. The product was identified by enzymatic digests followed by TLC analysis as described for pApU and as shown in Figure 4. The product was not hydrolyzed by bacterial alkaline phosphatase. Digestion with snake venom phosphodiesterase gave *p*-azidophenyl phosphate, AMP, and UMP. Digestion with both snake venom phosphodiesterase and bacterial alkaline phosphatase gave *p*-azidophenol, adenosine, and uridine. As shown in Figure 3, the 360-MHz proton NMR spectrum of the product had, in addition to the resonances for adenine and uridine, a clean AB pattern centered at 6.8 ppm corresponding to the para-substituted benzene ring. The yield of pure product was 5.9 μmol, determined by tritium radioactivity.

β-(4-Azidophenyl) Adenosine 5′-Diphosphate (N_3PhppA). A sample of 333 μmol of the acid form of AMP was converted to the mono[methyl(tri-*n*-octyl)ammonium] salt (Barker et al., 1972) and taken to dryness under reduced pressure. The AMP salt was then taken up 3 times in dry DMF and the solvent was removed under reduced pressure. A DMF solution of *p*-azidophenyl phosphorimidazolidate (65 μmol, 380 μL) was added to the methyl(tri-*n*-octyl)ammonium salt of AMP dissolved in 2 mL of dry DMF. The reaction was allowed to proceed 5 days in the dark. Solvent was removed under reduced pressure and the residue was taken up in water and methanol. The resulting homogeneous solution was applied to a 1 × 24 cm Sephadex A-25 column, acetate form. The column was eluted with an 800 mL gradient of 0.1–1.0 M ammonium acetate, pH 5.9. The absorbance at 310 nm of every other fraction was determined; the product eluted between 0.49 and 0.57 M salt. Solvent and excess salt were removed by rotary evaporation. The product had R_f 0.1 on PEI–cellulose TLC in solvent B. The presence of an azide moiety was demonstrated by the photoinduced browning of the product spot on the TLC plate after exposure to UV light. A 100-MHz NMR spectrum of the product showed, in addition to the resonances associated with the adenosine protons, an AB pattern characteristic of a para-substituted benzene ring, centered at 6.8 ppm. Enzymatic digests of the product were followed by TLC as described for the preparation of pApU. Digestion of the product with snake venom phosphodiesterase gave AMP and *p*-azidophenyl phosphate. The product was not hydrolyzed by bacterial alkaline phosphatase. Digestion with both bacterial alkaline phosphatase and snake venom phosphodiesterase gave adenosine and *p*-azidophenol. The product, β-(4-azidophenyl) adenosine 5′-diphosphate, had R_f 0.57 on silica gel TLC using solvent A. The yield was determined by reading the absorbance of a solution of the product at 260 nm and assuming that the molar extinction coefficient of the product was equal to the sum of the molar extinction coefficients of N_3Php and AMP (Maasen & Möller, 1974). When a molar extinction coefficient of 2.56×10^4 M^{-1} cm^{-1} was used, the yield of N_3PhppA was determined to be 25.9 μmol (40%).

Enzyme Assays. A modification of the dinucleotide assays of Downey & So (1970) was used. Each assay point contained the following in a total volume of 100 μL: 80 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 25 μM each ATP and UTP, 4.4 μg of poly[d(A-T)], 10 μg of *E. coli* RNA polymerase, and 1 mM K_2HPO_4. The concentration of the dinucleotide was varied from 2 to 50 μM. Stimulation of poly(A-U) synthesis was measured by using [α-^{32}P]ATP (0.067 μCi/assay). Inhibition of ATP incorporation in the 5′ end of poly(A-U) was measured by using [γ-^{32}P]ATP (1.4 μCi/assay) as the radiolabel. Assays were incubated in the dark at 37 °C for 10 min, followed by precipitation of nucleic acids by addition of 1 mL of 5% (w/v) trichloroacetic acid containing 10 mM sodium pyrophosphate. After standing 30 min at 0 °C, precipitates were collected on Millipore type HA nitrocellulose filters which had been soaked in the trichloroacetic acid–sodium pyrophosphate solution for at least 1 h at room temperature. Filters and precipitates were rinsed 3 times with 10 mL of 5% (w/v) trichloroacetic acid and 10 mM sodium pyrophosphate, dried 3 h under an IR lamp, and counted after addition of 5 mL of Aquasol.

Results and Discussion

Synthesis of Adenylyl-(3′–5′)-uridine 5′-Phosphate (pApU) by the Abortive Initiation Reaction. In order to prepare the dinucleotide photoaffinity label N_3PhppApU, it was necessary to synthesize micromolar quantities of the dinucleotide precursor pApU. Early efforts were made with the polynucleotide kinase catalyzed 5′-phosphorylation of commercially available ApU; this reaction was not found to be productive on a micromolar scale.

The abortive initiation reaction catalyzed by *E. coli* RNA polymerase in the presence of poly[d(A-T)] template and the substrates AMP and UTP gave pApU in high yield (74%). The concentrations of NaCl and $MgCl_2$ were chosen to optimize the abortive initiation reaction (Shemyakin et al., 1978; McClure et al., 1978). Inorganic pyrophosphatase was added to drive the reaction forward. Poly[d(A-T)] was present in

a limited amount (23 base pairs/RNA polymerase); titration of poly[d(A-T)] with *E. coli* RNA polymerase has indicated that 70 base pairs/polymerase might work better (Hansen & McClure, 1979). Product and nucleoside monophosphates were separated by anion-exchange chromatography at pH 5. At this pH, the nucleoside monophosphates should have a charge of −1 and the product a charge of −2. Proton NMR spectroscopy and enzymatic digests confirmed that the product isolated was pApU.

The abortive initiation reaction could conceivably be used to prepare other RNA fragments of defined sequence, complementary to a DNA template. Starting with dinucleotides, single bases have been added to generate triribonucleotides on synthetic and natural DNA templates (Oen & Wu, 1978; Oen et al., 1979). Recently, abortive initiation at the *lac* UV5 promoter has been shown to generate oligoribonucleotides up to six bases long (Capousis & Gralla, 1980).

p-Azidophenyl Phosphorimidazolidate. The preparation of *p*-azidophenyl phosphate from commercially available *p*-nitrophenyl phosphate was straightforward. Once the azide had been prepared, precautions were taken to handle it only under reduced light or in total darkness. Anion-exchange chromatography was used to isolate the product from side products which may have formed in the presence of the diazonium salt. The imidazolidate of *p*-azidophenyl phosphate was formed quantitatively, and is stable for at least a month if stored in anhydrous DMF at −79 °C in the dark. Once the imidazolidate is prepared, it can be used to couple the *p*-azidophenyl phosphate moiety to a variety of compounds bearing a phosphate group. It has been shown that the phosphorimidazolidate does not react with a phosphodiester linking two nucleotides, nor is reaction with the α-phosphate of a nucleoside diphosphate to give a branched nucleoside triphosphate likely (Kozarich et al., 1973; Hoard & Ott, 1965). Because of this selectivity, the phosphorimidazolidate of *p*-azidophenyl phosphate can be used to couple a photoaffinity probe specifically to terminal phosphates. The resulting diphosphates, unlike phosphoramidates, are stable under the acidic conditions required for visualizing proteins in polyacrylamide gels (Grachev & Zaychikov, 1974).

β-(4-Azidophenyl) Adenylyl-(3′–5′)-uridine 5′-Diphosphate (N₃PhppApU). N_3PhppApU was prepared by coupling the dinucleotide pApU with *p*-azidophenyl phosphorimidazolidate as shown in Figure 1. A possible side product of the coupling reaction is the symmetrical diester α,β-bis(4-azidophenyl) pyrophosphate (N_3PhppPhN_3), formed by reaction of *p*-azidophenyl phosphorimidazolidate with *p*-azidophenyl phosphate generated by hydrolysis. For separation of N_3PhppApU, pApU, *p*-azidophenyl phosphate, and N_3PhppPhN_3, an anion-exchange column was run at pH 5 where the compounds present should have charges of −3, −2, −1, and −2, respectively (Figure 2A). However, with the conditions used an impurity (apparently N_3PhppPhN_3) coeluted with the product N_3PhppApU. Separation by anion exchange at pH 2.5 (Figure 2B) followed by removal of salt gave pure product.

The aromatic region of the 360-MHz proton NMR spectrum of N_3PhppApU is shown in Figure 3. The proton resonance corresponding to H-8 on adenine is missing, probably due to coordination of a trace paramagetic metal ion impurity such as Cu^{2+} with the adenine moiety (Eichhorn et al., 1966). The resonances corresponding to H-2 on adenine and H-5 and H-6 on uracil as well as the resonances associated with the protons on the para-substituted benzene ring are all present with the expected intensities.

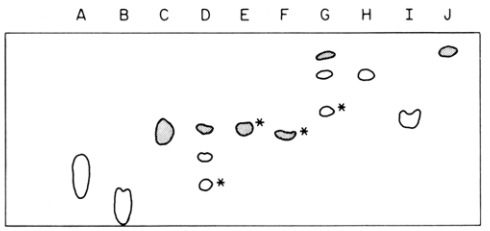

FIGURE 4: A tracing of the silica gel TLC plate (run in solvent A) for analysis of enzymatic digests of N_3PhppApU. Shading indicates a photosensitive spot and an asterisk indicates tritium activity. (A) AMP, (B) UMP, (C) *p*-azidophenyl phosphate, (D) snake venom phosphodiesterase digest of N_3PhppApU, yielding N_3Php, AMP, and UMP, (E) N_3PhppApU, (F) bacterial alkaline phosphatase digest of N_3PhppApU, (G) snake venom phosphodiesterase and bacterial alkaline phosphatase digest of N_3PhppApU, yielding N_3PhOH, A, and U, (H) adenosine, (I) uridine, (J) bacterial alkaline phosphatase digest of *p*-azidophenyl phosphate yielding N_3PhOH.

Figure 4 shows the thin-layer chromatogram of the enzymatic digests of the product. Photosensitivity and tritium radioactivity were monitored in addition to fluorescence quenching. More than 92% of the radioactivity was found associated with the expected uridine compound for each enzymatic digest. Double digests, with both bacterial alkaline phosphatase and snake venom phosphodiesterase, were done because the TLC system used resolved adenosine and uridine better than the corresponding monophosphates and because the product N_3PhppApU had the same R_f as *p*-azidophenyl phosphate. The enzymatic digests, coupled with the 360-MHz proton NMR spectrum, clearly demonstrate that the product is N_3PhppApU.

β-(4-Azidophenyl) Adenosine 5′-Diphosphate (N₃PhppA). Synthesis of N_3PhppA by coupling AMP with *p*-azidophenyl phosphorimidazolidate was straightforward. The product was separated from AMP, N_3Php, and N_3PhppPhN_3 by chromatography on DEAE-Sephadex at pH 5.0. NMR spectrocopy (100 MHz; data not shown) and TLC analysis of the product and its enzymatic digests confirmed that the product was pure N_3PhppA.

Inhibition and Stimulation of RNA Polymerase Activity with the Photoaffinity Probes N₃PhppApU and N₃PhppA. The photoaffinity probes N_3PhppApU and N_3PhppA are both diphosphates and are therefore not substrates for the elongation reaction catalyzed by RNA polymerase. However, they may be incorporated in the 5′ end of a nascent RNA chain. Downey & So (1970) have shown that incorporation of a dinucleotide in the 5′ end of an RNA chain can be demonstrated by observing stimulation of RNA synthesis and inhibition of [γ-^{32}P]ATP incorporation in the 5′ end of nascent RNA. The results of stimulation and inhibition studies done with N_3PhppApU, ApU, and N_3PhppA are shown in Figure 5. Both stimulation of poly(A-U) synthesis and inhibition of [γ-^{32}P]ATP incorporation were observed with the dinucleotide photoaffinity label N_3PhppApU, demonstrating that incorporation of the photoaffinity label in the 5′ end of nascent RNA does occur. The results also indicate that N_3PhppApU has a smaller apparent K_m than ApU; this may be due to the two additional negative charges on N_3PhppApU.

No stimulation of RNA synthesis was observed with the mononucleotide photoaffinity label N_3PhppA. Inhibition of [γ-^{32}P]ATP incorporation was observed, indicating competition of N_3PhppA with ATP for the initiation of RNA synthesis. Studies done with a number of adenosine-containing compounds bearing 5′ substituents have demonstrated their incorporation in the 5′ end of RNA chains (Armstrong & Eckstein, 1976; Yarbrough et al., 1979; Malygin & Shemyakin, 1979). These studies coupled with the results of the

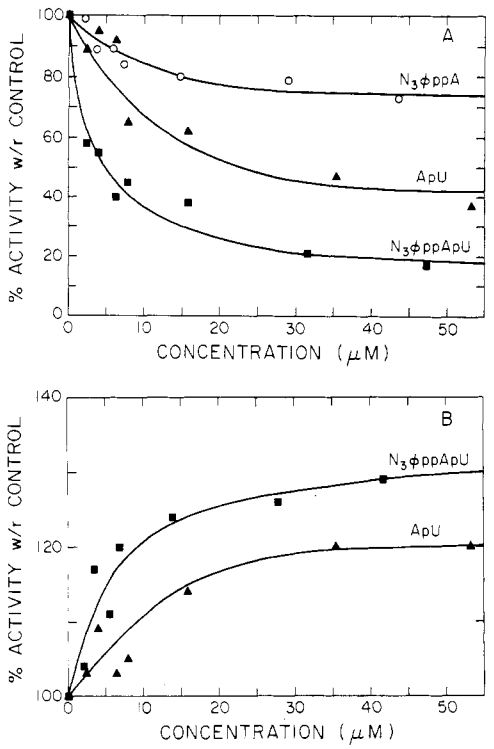

FIGURE 5: Effect of N_3PhppApU, ApU and N_3PhppA on chain initiation and elongation. The incubation mixtures were as described under Methods. The control reaction did not contain any of the initiators listed below. (A) Effect on initiation with [γ-^{32}P]ATP: ApU (▲), N_3PhppApU (■), N_3PhppA (○). (B) Stimulation of elongation: ApU (▲), N_3PhppApU (■). No stimulation was observed with N_3PhppA.

inhibition experiment (Figure 4A) indicate that N_3PhppA can also substitute for ATP in the 5′ end of nascent RNA.

Conclusions

The photoaffinity probes N_3PhppA and N_3PhppApU provide a useful means of specifically incorporating an aryl azide in the 5′ end of nascent RNA. Incubation of RNA polymerase with a DNA template of defined sequence and the appropriate combination of nucleoside triphosphates can be used to prepare a ternary DNA·enzyme·RNA complex containing RNA of known length. Using N_3PhppA and N_3PhppApU, we have prepared ternary complexes with RNA two, three, or four nucleotides long; photolysis followed by isolation of the individual enzyme subunits was used to determine the path of RNA across the surface of RNA polymerase in the first stages of transcription (DeRiemer & Meares, 1981).

The dinucleotide N_3PhppApU is a particularly efficient initiator of RNA synthesis. This compound and N_3PhppA are not substrates for the elongation of RNA; also their photoproducts are stable to the acidic conditions used for staining and destaining polyacrylamide gels. These advantages are not shared by the mononucleotide phosphoramidate photoaffinity probes prepared by Sverdlov & co-workers (1978, 1979).

The approach used here can be extended to incorporation of spectroscopic probes such as fluorescent dyes or metal chelates, to provide additional information about the environment at the 5′ terminus of RNA in the transcription complex.

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Early Steps in the Path of Nascent Ribonucleic Acid across the Surface of Ribonucleic Acid Polymerase, Determined by Photoaffinity Labeling†

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ABSTRACT: The photoaffinity probes β-(4-azidophenyl) adenosine 5′-diphosphate (N_3PhppA) and β-(4-azidophenyl) adenylyl-(3′–5′)-uridine 5′-diphosphate (N_3PhppApU) were used to determine the RNA polymerase subunit contacts made by the 5′ ends of three nascent RNA chains. Ternary enzyme·poly[d(A-T)]·oligonucleotide complexes were prepared in which the nascent oligonucleotide contained a photoaffinity label at the 5′ end and a ^{32}P radiolabel only at the 3′ end. The length of the RNA was fixed at two, three, or four nucleotides. Photolysis of the ternary complexes was followed by dissociation, polyacrylamide gel electrophoresis, autoradiography, and scintillation counting. With a dinucleotide probe, the enzyme subunits labeled were β′ (71%) and σ (21%). Photolysis of the ternary complex containing trinucleotide RNA also resulted in labeling of the β′ (64%) and σ (35%) subunits. With a tetranucleotide, the β′ subunit was very heavily labeled (88%), and a small amount of labeling of the β (7%) and σ (4%) subunits was observed. The α subunit was not labeled with any of the probes. These results imply that a conformational change, possibly involving dissociation of the σ subunit, occurs in the enzyme as the ribonucleotide is elongated from a tri- to a tetranucleotide.

Transcription of DNA is catalyzed by the oligomeric enzyme RNA polymerase with nucleoside triphosphates as substrates. The holoenzyme from *Escherichia coli* contains five subunits, denoted α,α,β′,β, and σ, with a total molecular weight of approximately 457 000 (Lowe et al., 1979). The σ subunit is required for proper initiation of RNA synthesis and dissociates at some point during elongation (Travers & Burgess, 1969). The functions of the other subunits are not yet known in as much detail (Zillig et al., 1976).

One basic question to be answered is which enzyme subunits come into contact with the leading (5′) end of the newly formed RNA molecule as it becomes elongated. Two unusual properties of RNA polymerase provide a way to experimentally determine this: after initiation of RNA synthesis, the ternary enzyme·DNA·RNA complex can be isolated and studied (Rhodes & Chamberlin, 1974; Rohrer & Zillig, 1977), and the 5′ terminal residue of the nascent RNA may contain a bulky 5′ substituent such as a photoaffinity label (Sverdlov et al., 1978; Yarbrough et al., 1979; Malygin & Shemyakin, 1979).

The two photoaffinity labels, β-(4-azidophenyl) adenosine 5′-diphosphate (N_3PhppA)[1] and β-(4-azidophenyl) adenylyl-(3′–5′)-uridine 5′-diphosphate (N_3PhppApU), have been prepared as described in the preceding paper (DeRiemer & Meares, 1981). By use of these probes, enzyme·DNA·RNA complexes containing RNA of precisely known length (two, three, or four bases), with a photoaffinity label on the 5′-phosphate and a radiolabel only on the 3′ end, have been prepared. Aryl azide photoaffinity probes were used because they are not chemically reactive until activated by photolysis, and the nitrenes formed by photolysis react rather indiscriminately with nearby molecules (Bayley & Knowles, 1977; Knowles, 1971). Initiation of RNA synthesis using a dinucleotide photoaffinity label (N_3PhppApU) proved to be very advantageous since initiation with mononucleotides requires very high concentrations of the affinity label. Photolyzed enzyme·DNA·RNA complexes were disrupted in sodium dodecyl sulfate ($NaDodSO_4$) and fractionated by gel electrophoresis. Finally autoradiography and scintillation counting revealed the distribution of radiolabel among enzyme subunits as a function of RNA chain length.

Experimental Procedures

Materials

All reagents and solvents were reagent grade and used without further purification unless noted otherwise. *N,N′*-Diallyltartardiamide was from Aldrich. Tris and Coomassie Brilliant Blue R-250 were from Sigma. Tris was recrystallized from methanol prior to use. Mononucleotides were from Sigma or P-L Biochemicals, Inc. Nucleoside [α-^{32}P]tri-

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[1] Abbreviations used: DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; N_3PhppA, β-(4-azidophenyl) adenosine 5′-diphosphate; N_3PhppApU, β-(4-azidophenyl) adenylyl-(3′–5′)-uridine diphosphate.

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