$$M_{L_2-L_1} = [ED]_0(e^{-k_{off}^{ss}t_2} - e^{-k_{off}^{ss}t_1})$$

Introducing the expression for polymer lengths results in the following equation relating M to the length distribution:

$$M_{L_2-L_1} = ED_0(e^{-k_{off}^{ss}L_2/k_{cat}} - e^{-k_{off}^{ss}L_1/k_{cat}})$$

The mean length predicted is $\bar{M}$ = 40 nucleotides for k_{cat} = 4 s^{-1} and k_{off}^{ss} = 0.1 s^{-1}.

Registry No. Pol I, 9012-90-2; MgTTP, 72781-90-9.

References

Bambara, R. A., Uyemura, D., & Lehman, I. R. (1976) *J. Biol. Chem. 251*, 4090.

Bambara, R. A., Uyemura, D., & Choi, T. (1978) *J. Biol. Chem. 253*, 413.

Benkovic, P. A., Bullard, W. P., deMaine, M. M., Fishbein, R., Schray, K. J., Steffens, J. J., & Benkovic, S. J. (1974) *J. Biol. Chem. 249*, 930.

Benkovic, S. J., & Schray K. J. (1971) *Enzymes, 3rd Ed. 8*, 201.

Berger, R. L., Balko, B., & Chapman, H. (1968) *Rev. Sci. Instrum. 39*, 493.

Brutlag, D., & Kornberg, A. (1972) *J. Biol. Chem. 247*, 241.

Bryant, R., & Benkovic, S. J. (1983) *Anal. Biochem.* (in press).

Burgers, P. M., & Eckstein, F. (1980) *J. Biol. Chem. 254*, 6889.

Das, S. K., & Fujimura, R. K. (1979) *J. Biol. Chem. 254*, 1227.

Das, S. K., & Fujimura, R. K. (1980) *J. Biol. Chem. 249*, 841.

Davis, R. W., Botstein, D., & Roth, J. R. (1980) *Advanced Bacterial Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Dyson, R. D., & Isenberg, I. (1971) *Biochemistry 10*, 3233.

Englund, P. T., Huberman, J. A., Jovin, T. M., & Kornberg, A. (1969) *J. Biol. Chem. 244*, 3038.

Gutfreund, H. (1972) *Enzymes: Physical Principles*, Wiley-Interscience, New York.

Johnson, K. A. (1983) *J. Biol. Chem.* (in press).

Jovin, T. M., Englund, P. T., & Bertsch, L. L. (1969a) *J. Biol. Chem. 244*, 2996.

Jovin, T. M., Englund, P. T., & Kornberg, A. (1969b) *J. Biol. Chem. 244*, 3009.

Knowles, J. (1980) *Annu. Rev. Biochem. 49*, 877.

Kornberg, A. (1980) *DNA Replication*, W. H. Freeman, San Francisco, CA.

Lynn, R. W., & Taylor, E. W. (1970) *Biochemistry 9*, 2975.

McClure, W. R., & Jovin, T. M. (1975) *J. Biol. Chem. 250*, 4073.

McClure, W. R., & Chow, Y. (1980) *Methods Enzymol. 64*, 277.

Porter, M. E., & Johnson, K. A. (1983) *J. Biol. Chem.* (in press).

Rose, I. A. (1980) *Methods Enzymol. 64*, 47.

Travaglini, E. C., Mildvan, A. S., & Loeb, L. A. (1975) *J. Biol. Chem. 250*, 8647.

Wang, T. S.-F., Sedwick, W. D., & Korn, D. (1974) *J. Biol. Chem. 249* 841.

Wilkinson, K. D., & Rose, I. A. (1979) *J. Biol. Chem. 254*, 12567.

Synthesis of a Cleavable Dinucleotide Photoaffinity Probe of Ribonucleic Acid Polymerase: Application to Trinucleotide Labeling of an *Escherichia coli* Transcription Complex†

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ABSTRACT: The cleavable dinucleotide photoaffinity probe 5′-[[(4-azidophenacyl)thio]phosphoryl]adenylyl(3′–5′)uridine was prepared and used to determine the 5′ contacts of a trinucleotide in an *Escherichia coli* RNA polymerase/T7 DNA transcription complex. The probe was prepared by alkylating 5′-(thiophosphoryl)adenylyl(3′–5′)uridine with azidophenacyl bromide. The 5′-(thiophosphoryl)adenylyl(3′–5′)uridine was prepared by the abortive initiation reaction of RNA polymerase on a poly[d(A-T)] DNA template, using adenosine 5′-*O*-(thiomonophosphate) and uridine triphosphate as substrates. A transcription complex containing a radiolabeled trinucleotide at the A1 promoter of bacteriophage T7 D111 or D123 DNA was prepared by using the dinucleotide photoaffinity probe as initiator and cytidine [α-^{32}P]triphosphate as the other substrate. After photolysis, the labeled subunits and DNA were isolated, and the trinucleotide was removed in the presence of phenylmercuric acetate and analyzed by polyacrylamide gel electrophoresis. The 5′ end of the trinucleotide was found to label the DNA (≈88%) and also the β (≈10%) and σ (≈3%) subunits of *E. coli* RNA polymerase. It was also shown that the order of migration of the β and β' subunits of *E. coli* RNA polymerase on polyacrylamide gel electrophoresis in sodium dodecyl sulfate is different from that in sodium dodecyl sulfate plus urea.

RNA polymerase is an oligomeric enzyme which catalyzes the synthesis of RNA, using DNA as a template. *Escherichia coli* RNA polymerase holoenzyme contains five major subunits with a total molecular weight of approximately 454 000 and consists of subunits β' (M_r 160 000), β (M_r 151 000), σ (M_r 70 000), and (two) α (M_r 36 000) (Chamberlin, 1982). The σ subunit is required for specific initiation of transcription at promoter sites on DNA and dissociates shortly thereafter to leave the core enzyme (Travers & Burgess, 1969; Hansen & McClure, 1980). Less is known about the functions of the other enzyme subunits.

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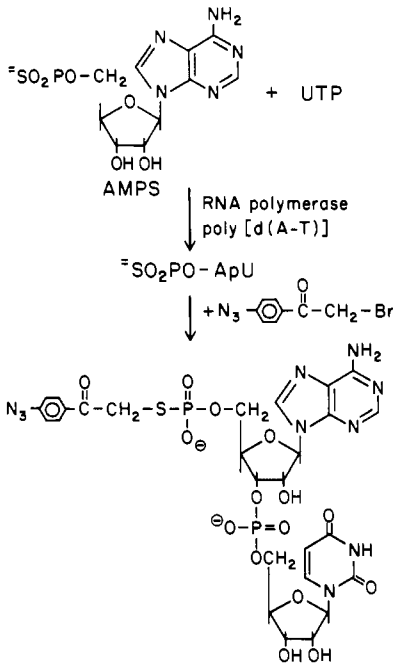

FIGURE 1: Synthesis of 5′-[[(4-azidophenacyl)thio]phosphoryl]-adenylyl(3′–5′)uridine (N_3RSpApU). The dinucleotide SpApU (SO_2PO-ApU) is prepared by the abortive initiation reaction catalyzed by RNA polymerase in the presence of AMPS, UTP, and poly[d-(A-T)]. The SpApU is then alkylated with azidophenacyl bromide to give N_3RSpApU (R = $-C_6H_4COCH_2-$).

RNA synthesis occurs by polymerization of nucleoside triphosphates in a 5′ to 3′ direction. Dinucleotides have been shown to be more efficient initiators of transcription than mononucleotides (Downey & So, 1970), and the 5′-terminal residue of the RNA may contain a bulky substituent (Yarbrough et al., 1979; Malygin & Shemyakin, 1979) such as a photoaffinity label. Taking advantage of these facts, we have prepared the cleavable dinucleotide photoaffinity probe 5′-[[(4-azidophenacyl)thio]phosphoryl]adenylyl(3′–5′)uridine (N_3RSpApU),[1] shown that it specifically initiates transcription at the A1 promoter of bacteriophage T7 DNA, and used it to determine the DNA and RNA polymerase subunit contacts of the 5′ end of a trinucleotide.

The dinucleotide probe (Figure 1) contains a photoreactive aryl azide connected to the 5′-phosphate by an S–P bond. The aryl azide was chosen because it is not chemically reactive until irradiated with ultraviolet light, and the nitrene generated thereby reacts relatively indiscriminately with its surroundings (Knowles, 1971; Bayley & Knowles, 1977) to cause covalent attachment to DNA or protein. The S–P linkage was included because such bonds can be selectively hydrolyzed at neutral pH and room temperature in the presence of organomercurials (Akerfeldt, 1959; Neumann & Smith, 1967). This allows release of the label from photoaffinity-labeled molecules, when desired. The probe was easily prepared by alkylation with azidophenacyl bromide of the dinucleotide SpApU, which was prepared by the abortive initiation reaction of *E. coli* RNA polymerase on a poly[d(A-T)] template (DeRiemer & Meares, 1981a).

N_3RSpApU was used to initiate trinucleotide synthesis at the A1 promoter of both T7 D111 and D123 DNA, from which the RNA transcripts begin 5′-AUCG. The trinucleotide was radiolabeled by the addition of [α-^{32}P]CTP to the 3′-position of the dinucleotide initiator. Further elongation to the tetranucleotide was prevented by the absence of GTP in the reaction mixture. After covalent attachment of the 5′ end of the trinucleotide to adjacent macromolecules, the components of the transcription complex were separated by polyacrylamide gel electrophoresis and recovered by electroelution from the gel. The DNA and RNA polymerase subunits were treated with phenylmercuric acetate, and after a second gel electrophoresis, the amount of trinucleotide released from each subunit or the DNA was measured by densitometer scanning of the autoradiogram.

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*-Azidophenacyl bromide was purchased from Pierce. Adenosine 5′-*O*-(thiomonophosphate) (AMPS) was from Boehringer-Mannheim. Poly[d(A-T)], nucleotides, bacterial alkaline phosphatase, and snake venom phosphodiesterase were from Sigma. [α-^{32}P]CTP and [^{3}H]UTP were from Amersham. Silica gel 60 F_{254} thin-layer chromatography plates were from Merck. *E. coli* MRE 600 cells were from Grain Processing Corp.

Buffers and Solvents. The buffers used were as follows: buffer A, 0.25 M Tris-HCl, pH 7.9, 0.025 M 2-mercaptoethanol, 0.05 M NaCl, 0.05 M $MgCl_2$, 25% (v/v) glycerol, and 5 mM K_2HPO_4; buffer B, 8 M urea, 50% (w/v) sucrose, 1.2% (w/v) $NaDodSO_4$, 0.15% (w/v) bromphenol blue, 70 mM triethanolamine, and 50 mM HCl, pH 7.5; buffer C, 0.44% (v/v) ethanolamine, 0.45% (w/v) glycine, and 0.15% (w/v) $NaDodSO_4$, pH 9.7; buffer D, 0.01 M Tris-HCl, pH 8.0, and 1 mM EDTA; buffer E, 80 mM Tris-HCl, pH 7.9, 5 mM 2-mercaptoethanol, 50% (v/v) glycerol, and 0.1 mM EDTA; buffer F, 0.11 M Tris-HCl, pH 8.9, and 0.11 M NaCl; buffer G, 1.9 M Tris-HCl, pH 8.0.

The solvents used were as follows: solvent H, 2-propanol/concentrated ammonium hydroxide/water (6:3:1 v/v/v); solvent I, 1% (w/v) ammonium acetate, pH 7.0; solvent J, 1% (w/v) ammonium acetate, pH 5.0; solvent K, 0.8% (w/v) ammonium acetate, pH 7.0, and 20% (v/v) acetonitrile.

Methods

Thin-Layer Chromatography. Product structure was verified by analysis on silica gel F_{254} TLC plates. Three-microliter sample aliquots were applied 1 cm above the bottom of the TLC plate, and the solvent front was allowed to move at least 11 cm up the plate. Aromatic compounds were visualized by fluorescence quenching. Photosensitive compounds were located by irradiating the TLC plate in a Rayonet-type RS photochemical reactor ($\lambda > 300$ nm) for 3 min. Radioactive spots were located by scraping the silica gel from the TLC plate and suspending in Aquasol scintillation cocktail for counting.

DNA Templates. Bacteriophage T7 D123 DNA (M_r 25.5 $\times 10^6$) and bacteriophage T7 D111 were kindly provided by Judy Levin and Michael Chamberlin. The D111 DNA was isolated by $NaDodSO_4$–KCl extraction of protein (Schleif & Wensink, 1981). Both T7 DNA and poly[d(A-T)] were dialyzed into buffer D. Concentrations were determined by measuring the absorbance at 260 nm (Schleif & Wensink, 1981), and the DNA was stored at 4 °C.

RNA Polymerase. This was isolated from *E. coli* MRE 600 cells by using the method of Burgess & Jendrisak (1975) with

[1] Abbreviations: SpApU, 5′-(thiophosphoryl)adenylyl(3′–5′)uridine; N_3RSpApU, 5′-[[(4-azidophenacyl)thio]phosphoryl]adenylyl(3′–5′)-uridine; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; AMPS, adenosine 5′-*O*-(thiomonophosphate).

the modifications of Lowe et al. (1979). The enzyme was dialyzed into buffer E and stored at −79 °C in 5-μL aliquots. Protein concentrations were determined by the method of Bradford (1976) and Sedmak & Grossberg (1977).

Synthesis of 5′-(Thiophosphoryl)adenylyl(3′–5′)uridine (SpApU). The abortive initiation reaction catalyzed by RNA polymerase (Johnston & McClure, 1976) was used to synthesize SpApU. The method was the same as for pApU synthesis (DeRiemer & Meares, 1981a), except that AMPS was used as initiator (Figure 1) instead of AMP. The reaction mixture contained the following in 10 mL: 20 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 5.3 mM AMPS, 4.5 mM UTP (10 μCi/mL [^{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. The reaction mixture was purified by high-pressure liquid chromatography on a Waters semipreparative C_{18} μBondapak column with solvent I. With a solvent flow rate of 5 mL/min, the product had an 8-min retention time. Ammonium acetate was removed by lyophilization. The product had an R_f value of 0.34 on TLC in solvent H. Concentrations were determined by reading the absorbance at 260 nm, using $\epsilon_{260} = 2.5 \times 10^4$ M^{-1} cm^{-1}.

Synthesis of 5′-[[(4-Azidophenacyl)thio]phosphoryl]-adenylyl(3′–5′)uridine (N_3RSpApU). Synthesis of the dinucleotide photoaffinity probe was carried out under reduced light. One hundred microliters of 13 mM SpApU and 10 μL of 0.2 M $NaHCO_3$ were mixed with 30 μL of methanol; 30 μL of 90 mM azidophenacyl bromide in methanol was then added, and the reaction was allowed to proceed for 45 min at room temperature. The reaction mixture was extracted 3 times each with isobutyl alcohol and then with ethyl ether, and ether was removed under reduced pressure at room temperature. The reaction mixture was then applied to the Waters HPLC column and was eluted with a 20-min linear gradient from solvent J to solvent K. With a solvent flow rate of 2 mL/min, the product had a retention time of 19–20 min. Acetonitrile was removed with three ether extractions, and the solvent was then removed by lyophilization. The ultraviolet spectrum of each peak from the HPLC column was taken on a Hewlett-Packard Model 8450 UV–vis spectrophotometer. Product concentration was determined by using $\epsilon_{300} = 2 \times 10^4$ M^{-1} cm^{-1} for the azide (Hixson & Hixson, 1975). The product had an R_f of 0.56 on TLC in solvent H.

Enzyme Digestion and S–P Bond Cleavage Reactions. The product structure was verified by enzymatic digestion and mercury-facilitated cleavage of the N_3RSpApU followed by analysis on TLC in solvent H (Figure 2). Enzyme digests were done at 37 °C for 1 h. The snake venom phosphodiesterase and bacterial alkaline phosphatase digestion reactions each contained 10 μL of 1 mM N_3RSpApU, 1 unit of enzyme, and 10 μL of buffer E or F, respectively. The double digest contained 10 μL of 1 mM N_3RSpApU, 1 unit each of snake venom phosphodiesterase and bacterial alkaline phosphatase, and 5 μL each of buffers E and F. Hydrolysis of the S–P bond was carried out by adding 10 μL of saturated phenylmercuric acetate in 0.1% (w/v) $NaDodSO_4$ to 10 μL of 1 mM N_3RSpApU at neutral pH. Cleavage was complete after 12 h at room temperature. Both reactions and thin-layer chromatography were done in reduced light. The digestion and cleavage reactions were applied to the same TLC plate with the appropriate control compounds.

Trinucleotide Preparation. The structure of the probe was further verified by its ability to specifically initiate transcription at the A1 promoter of T7 DNA. The RNA transcript from this promoter begins with the sequence AUC. The reaction contained the following in 20 μL: 5 nM T7 D111 DNA, 10 nM *E. coli* RNA polymerase, 2 μL of buffer A, 100 μM N_3RSpApU, and 6 μM [α-^{32}P]CTP (410 mCi/μmol). The DNA, RNA polymerase, and N_3RSpApU were preincubated at 37 °C for 5 min before addition of [α-^{32}P]CTP. After 5 min, the reaction was stopped by adding 20 μL of saturated urea. Half of the reaction mixture was added to 10 μL of 20 mM dithiothreitol to reduce azide to amine, while the other half was added to 10 μL of saturated phenylmercuric acetate in 0.1% (w/v) $NaDodSO_4$ to hydrolyze the S–P bond. Samples were allowed to sit overnight at room temperature and were then adjusted to 10 mM dithiothreitol, 0.05% (w/v) bromphenol blue, and 0.05% (w/v) xylene cyanol for electrophoresis on polyacrylamide gels.

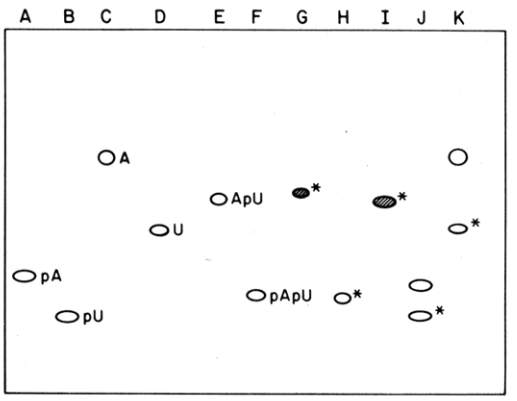

FIGURE 2: Tracing of the silica gel TLC plate (run in solvent H) for analysis of the enzymatic digests and hydrolysis of N_3RSpApU. Shading indicates a photosensitive spot, and an asterisk indicates the presence of a [^{3}H]uridine residue: (A) AMP; (B) UMP; (C) adenosine; (D) uridine; (E) ApU; (F) pApU; (G) N_3RSpApU; (H) hydrolysis of N_3RSpApU in phenylmercuric acetate, yielding pApU; (I) bacterial alkaline phosphatase digest of N_3RSpApU (no reaction); (J) snake venom phosphodiesterase digestion of N_3RSpApU, yielding AMP and UMP; (K) snake venom phosphodiesterase and bacterial alkaline phosphatase digestion of N_3RSpApU, yielding adenosine and uridine. In reactions H, J, and K, azide-containing cleavage products move with the solvent front.

Trinucleotide Labeling of E. coli RNA Polymerase on T7 D111 (or D123) DNA. The trinucleotide photoaffinity reaction contained the following in 80 μL: 10 nM T7 D111 (or D123) DNA, 20 nM *E. coli* RNA polymerase, 100 μM N_3RSpApU, 6 μM [α-^{32}P]CTP (410 mCi/μmol), and 8 μL of buffer A. Again, DNA, RNA polymerase, and dinucleotide intiator were preincubated in the dark at 37 °C for 5 min prior to addition of CTP. Five minutes afterward, the trinucleotide-containing sample was split into two 40-μL aliquots. One aliquot was irradiated for 2 min in a Rayonet-type RS photochemical reactor ($\lambda > 300$ nm) in a 6 × 20 mm borosilicate glass tube. The second aliquot was kept in the dark for 2 min. Transcription complexes in both were then disrupted by the addition of 10 μL of buffer B, and excess azide was reduced with 10 mM dithiothreitol. After the aliquots sat in the dark for 1 h at room temperature, the DNA and protein subunits were separated on either $NaDodSO_4$–urea–polyacrylamide (Wu & Bruening, 1971; Halling et al., 1977) or $NaDodSO_4$–polyacrylamide (Laemmli, 1970) gels; 0.7 mm thick, 40 cm long step-gradient gels were used in both systems. The $NaDodSO_4$–urea–polyacrylamide gels contained a 32-cm, 3.5% polyacrylamide upper layer and a 6-cm, 7% polyacrylamide lower layer and were run in buffer C. The $NaDodSO_4$–polyacrylamide gels without urea contained a 32-cm, 5% polyacrylamide upper layer and a 5-cm, 10% polyacrylamide lower layer. Both types of gels were electrophoresed at 25 mA

until the bromphenol blue marker reached the bottom of the gel. Control reactions, each containing the complete reaction minus DNA, N_3RSpApU, or RNA polymerase, were prepared and photolyzed in the same way as the complete photoaffinity reactions.

Electroelution and Cleavage. DNA and protein bands from the complete photolyzed reaction were excised from the step-gradient gels, placed in dialysis bags with 0.2 mL of 0.1% (w/v) $NaDodSO_4$, and electroeluted for 3 h at 50 mA. The solutions were removed from the dialysis bags, and 0.2 mL of saturated phenylmercuric acetate in 0.1% (w/v) $NaDodSO_4$ was added to each. Soluble RNA was added to a final concentration of 0.5 mg/mL. Cleavage of the trinucleotide from labeled components was complete after 24 h at room temperature. Samples were then lyophilized and resuspended in 40 μL of 7 M urea. Marker dyes were added, and 20-μL samples were loaded onto 25% polyacrylamide gels.

RNA Gel Electrophoresis. RNA transcripts were analyzed on 40-cm 25% polyacrylamide gels [0.089 M Tris–borate, 2.5 mM EDTA, 7 M urea, and 1:29 methylenebis(acrylamide):acrylamide]. Gels were allowed to polymerize 12 h and were then preelectrophoresed for 6 h at 1000 V before use. Ten-microliter aliquots were loaded, and electrophoresis proceeded at 1000 V until the bromphenol blue marker had migrated 27.5 cm from the bottom of the sample well. Autoradiography of gels was carried out at −79 °C with Kodak X-OMat AR-5 X-ray film and Cronex Lighting-Plus intensifying screens.

Results

5′-[[(4-Azidophenacyl)thio]phosphoryl]adenylyl(3′–5′)-uridine. N_3RSpApU was prepared by the alkylation of SpApU with azidophenacyl bromide, as shown in Figure 1. The isolation of SpApU has been greatly simplified by the use of HPLC instead of anion-exchange chromatography (DeRiemer & Meares, 1981a). The use of HPLC makes the removal of DNA and protein by ultrafiltration unnecessary, since these are retained on the HPLC guard column.

The alkylation reaction to give N_3RSpApU is rapid and nearly quantitative (yields are typically greater than 90%). We generally prepare small quantities and store these in the dark at −20 °C for up to 3 months. The product appears to be quite stable under these conditions, showing no changes in the ultraviolet spectrum or migration on TLC. Storage at −80 °C is recommended if the probe is to be kept longer than 3 months before use.

Product Structure Verification. Figure 2 shows the thin-layer chromatogram of the enzymatic digests and hydrolysis of N_3RSpApU. The nucleotide portion of the product is clearly resolved with this TLC system, and the cleavage products obtained are consistent with the probe structure N_3RSpApU. The azide portion of the product runs with the solvent front. The S–P bond evidently is cleaved by snake venom phosphodiesterase.

The product structure was further verified by its ability to specifically initiate RNA synthesis. When N_3RSpApU and [α-^{32}P]CTP are incubated with RNA polymerase and T7 D111 (or D123) DNA, the trinucleotide N_3RSpApUpC should be synthesized. Both of these DNAs contain only one strong *E. coli* RNA polymerase promoter, the A1 promoter, from which the RNA transcript begins 5′-AUCG. In the absence of GTP, the transcript cannot proceed beyond the trinucleotide. Trinucleotide synthesis on this promoter was initiated with N_3RSpApU or pApU in the presence of [α-^{32}P]CTP to give radiolabeled N_3SpApUpC and pApUpC, respectively. Hydrolysis of the S–P bond of N_3RSpApUpC

FIGURE 3: Autoradiogram of the 25% polyacrylamide gel showing that N_3RSpApU can specifically initiate transcription at the A1 promoter of T7 D111 DNA. A trinucleotide was prepared by incubating N_3RSpApU with [α-^{32}P]CTP in the presence of RNA polymerase and DNA. The first lane shows NH_2RSpApUpC prepared by dithiothreitol reduction of N_3RSpApUpC. The second lane shows pApUpC prepared by phenylmercuric acetate hydrolysis of N_3RSpApUpC. The third lane contains authentic pApUpC.

should give pApUpC. Reduction of the azide with dithiothreitol should give NH_2RSpApUpC. Figure 3 compares the electrophoretic behavior of the trinucleotides prepared. Reduction of the putative N_3RSpApUpC with dithiothreitol gave a product which migrated slower than pApUpC on the 25% gels. If the N_3RSpApUpC was treated with phenylmercuric acetate rather than dithiothreitol, complete hydrolysis of the S–P bond occurred to give a single product which migrated identically with the trinucleotide pApUpC. This mobility change is consistent with cleavage of the S–P bond of N_3RSpApUpC to give the smaller, more negatively charged pApUpC.

Photoaffinity Labeling by the Trinucleotide. The procedure used to determine the DNA and RNA polymerase contacts of the leading end of the trinucleotide is shown in Figure 4. Irradiation of the ternary transcription complex causes covalent attachment of the trinucleotide to nearby DNA or protein. Dissociation of the ternary complex with $NaDodSO_4$ and electrophoresis on polyacrylamide gels separate the protein subunits and DNA. Two types of gels have been used, and autoradiograms of both are shown in Figure 5. Protein subunits and DNA were located by silver staining. The gel in Figure 5A is the commonly used $NaDodSO_4$ system of Laemmli (1970) in which the β subunit moves just ahead of the larger β' subunit. It is clear from comparing subunit positions determined by silver staining to the location of radioactivity determined by autoradiography that the β and σ subunits were photoaffinity labeled. There was no radioactivity associated with these subunits in any of the control reactions. The DNA was also radiolabeled, but radiolabeling occurred even in the absence of irradiation or azide initiator because RNA polymerase catalyzes the addition of ribonucleotides to

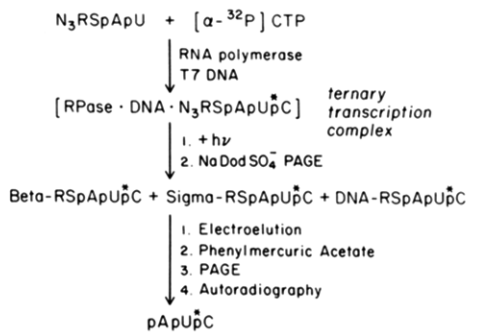

FIGURE 4: Procedure for the synthesis of N_3RSpApUpC and determination of DNA and RNA polymerase subunit contacts by the 5′ end of this trinucleotide. N_3RSpApU and [α-^{32}P]CTP are joined by RNA polymerase in the presence of T7 D111 (or D123) DNA. The resulting transcription complex can be irradiated ($\lambda > 300$ nm) to covalently attach the trinucleotide to nearby DNA and enzyme subunits, which are then separated by NaDodSO$_4$–polyacrylamide gel electrophoresis and recovered by electroelution from the gel. The trinucleotide is removed from labeled components by hydrolysis of the S–P bond in the presence of phenylmercuric acetate and quantitated by electrophoresis and autoradiography.

the ends of DNA (Nath & Hurwitz, 1974). Determination of photoaffinity labeling in this case required cleavage of the trinucleotide from the DNA. The gel in Figure 5B is the NaDodSO$_4$–urea system used in earlier trinucleotide experiments on poly[d(A-T)], in which it was reported that the β' and σ subunits of *E. coli* RNA polymerase were labeled (DeRiemer & Meares, 1981b). It is clear from comparing panels A and B of Figure 5 that the β and β' subunits are inverted in the NaDodSO$_4$–urea gel system relative to that in Laemmli gels. On the NaDodSO$_4$–urea gel, the radioactivity was associated with the slower moving of the two large subunits. This was verified by excising gel bands from both types of gels and scintillation counting. The subunit inversion was further verified by showing that the β' subunit, which binds to Affi-Gel Blue resin (Wu et al., 1977), is the faster moving subunit on the NaDodSO$_4$–urea gels (data not shown). These results thus show that it was β, rather than β', which was most heavily photoaffinity labeled in the experiments of DeRiemer & Meares (1981b).

Electroelution and Cleavage. After autoradiography, the DNA and protein subunits from the irradiated reaction were electroeluted from the gel pieces. Gel pieces were counted before and after electroelution, and more than 90% of the radioactivity could be retrieved if the gels were not stained and destained. The recovery dropped to about 50% and required longer electroelution times if gels were stained. After electroelution, phenylmercuric acetate was added to the DNA or protein subunits to release any attached trinucleotide by hydrolysis of the S–P bond. The hydrolysis was complete after 24 h at room temperature if fresh mercurial solutions were used. Once cleavage was complete, the samples were concentrated by lyophilization and resuspended for analysis on 25% acrylamide gels. Carrier RNA was added to decrease the loss of trinucleotide on container surfaces. The autoradiogram in Figure 6 shows the components contacted by the 5′ end of the trinucleotide. Both DNA templates (D111 and D123) gave practically the same results. The products cleaved from the DNA and the β and σ protein subunits have the same mobility as pApUpC (Figure 3). Of the total number of transcription complexes present in the sample, roughly 1% were photoaffinity labeled; the majority of this photoaffinity labeling occurred on the DNA (88 ± 5%), with β (10 ± 2%) and σ (3 ± 2%) also labeled. There was no detectable photoaffinity labeling of β' or α.

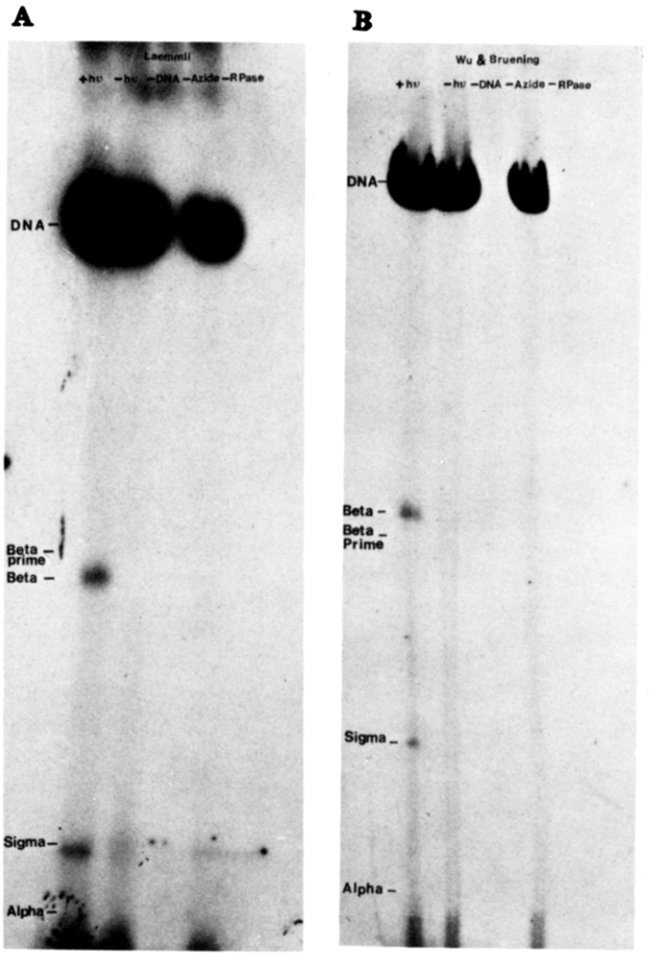

FIGURE 5: Autoradiograms of the step-gradient NaDodSO$_4$–polyacrylamide gels used to separate DNA and protein subunits after photoaffinity labeling (Figure 4). (A) "Laemmli" NaDodSO$_4$ gel; (B) "Wu & Bruening" (1971) NaDodSO$_4$–urea gel. Note that the order of mobilities of the β and β'subunits is different in these two gel systems. The left lane of each gel contains the complete trinuleotide photoaffinity reaction. The remaining lanes contain reactions lacking irradiation, DNA, N_3RSpApU, or RNA polymerase. DNA and polymerase subunit locations (noted at left) were determined by silver staining the gels. Note that the DNA is radiolabeled even in the absence of irradiation or N_3RSpApU, due to addition of [α-^{32}P]CTP to DNA ends by RNA polymerase. Nevertheless, photoaffinity labeling of the DNA may be measured after specific cleavage of the S–P bond (Figures 4 and 6).

Discussion

There have been several types of studies designed to determine which enzyme subunits comprise the active site of *E. coli* RNA polymerase. Most involve binding of radiolabeled, active site directed reagents to RNA polymerase in the presence or absence of DNA, followed by covalent attachment of these reagents to the polymerase and determination of the enzyme subunits labeled. When radiolabeled 5-formyluridine 5′-triphosphate was incubated with and covalently attached to RNA polymerase, the radioactivity was located entirely on the β subunit (Armstrong et al., 1976). When ^{32}P-labeled 4-thiouridine triphosphate was photochemically attached, both the β and β' subunits were labeled, with the majority of the label on the β' subunit (Frischauf & Scheit, 1973); however, in the presence of poly[d(A-T)], β was predominantly labeled. When covalent attachment of substrate analogues was through the 3′-position of the sugar, and DNA was included in the reaction mixture, only the β' subunit of the core enzyme was labeled (Armstrong & Eckstein, 1979). The differences observed in those studies are due both to the different reactive

FIGURE 6: Autoradiogram of a 25% polyacrylamide gel showing the relative amounts of pApUpC cleaved from each of the transcription components. Dashed curve indicates the shape of the tracking dye band.

sites on the substrate analogues and to the effect of DNA (covering sites on the polymerase or causing a change in its conformation). It seems clear, however, that both the β and β' subunits are involved in the catalytic center.

Studies which involve the synthesis and attachment of short oligonucleotides to RNA polymerase have shown that the σ subunit is involved also. Sverdlov et al. (1980) reported labeling the σ subunit with a nascent RNA containing a 5-iodouridine residue one position removed from the 3′ end. In light of the results in Figure 5, the β and σ subunits have been shown to be in contact with the 5′ ends of the dinucleotide and trinucleotide synthesized on a poly[d(A-T)] template (DeRiemer & Meares, 1981b). The β and σ subunits have also been shown to be in contact with the DNA template near the site at which RNA synthesis begins (Simpson, 1979).

Our findings that the 5′ end of the trinucleotide synthesized at the A1 promoter of T7 DNA contacts the β and σ subunits, as well as the DNA, are in good agreement with the other studies. There are certain advantages to our approach. Because the rate of abortive initiation is quite slow at the A1 promoter (Nierman & Chamberlin, 1979), the use of a nonradioactive photoaffinity probe avoids nonspecific labeling by free probe and allows only catalytically active polymerase molecules to be studied. The azide-containing dinucleotide must be joined to the [α-^{32}P]CTP by phosphodiester bond formation before photoaffinity labeling will be observed. In addition, due to the end-on addition of [α-^{32}P]CTP to the DNA template, DNA photoaffinity labeling can only be determined after cleavage of the S–P bond of the bound trinucleotide and separation from the end-labeled DNA. This procedure also confirms that photoaffinity labeling results from the trinucleotide only.

By extending this approach to photoaffinity labeling with oligonucleotides of many different lengths, we have determined the entire path of the leading end of nascent RNA through a transcription complex. In order to do this, it was necessary to address several technical problems not encountered with the trinucleotide; these include the production of detectable quantities of RNAs spanning a wide range of lengths (from 4 to 116 nucleotides), the unambiguous identification of RNAs involved in labeling, and the separation of β and β' subunits when labeled by long RNAs. The results of these experiments will be published separately (Hanna & Meares, 1983).

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References

Akerfeldt, S. (1959) *Acta Chem. Scand. 13*, 1479–1480.
Armstrong, V. W., & Eckstein, F. (1979) *Biochemistry 18*, 5117–5122.
Armstrong, V. W., Sternbach, H., & Eckstein, F. (1976) *Biochemistry 15*, 2086–2091.
Bayley, H., & Knowles, J. R. (1977) *Methods Enzymol. 46*, 69–114.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Burgess, R. R., & Jendrisak, J. J. (1975) *Biochemistry 14*, 4634–4636.
Chamberlin, M. J. (1982) *Enzymes, 3rd Ed. 15*, 61–82.
DeRiemer, L. H., & Meares, C. F. (1981a) *Biochemistry 20*, 1606–1612.
DeRiemer, L. H., & Meares, C. F. (1981b) *Biochemistry 20*, 1612–1617.
Downey, K. M., & So, A. G. (1970) *Biochemistry 9*, 2520–2525.
Frischauf, A. M., & Scheit, K. M. (1973) *Biochem. Biophys. Res. Commun. 53*, 1227–1233.
Halling, S. M., Burtis, K. C., & Doi, R. H. (1977) *J. Biol. Chem. 252*, 9024–9031.
Hanna, M. M., & Meares, C. F. (1983) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Hansen, U. M., & McClure, W. R. (1980) *J. Biol. Chem. 255*, 9564–9570.
Hixson, S. H., & Hixson, S. S. (1975) *Biochemistry 14*, 4251–4254.
Johnston, D. E., & McClure, W. R. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 413–428, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Knowles, J. R. (1971) *Acc. Chem. Res. 5*, 155–160.
Laemmli, U. (1970) *Nature (London) 227*, 680–685.
Lowe, P. A., Hager, E. A., & Burgess, R. R. (1979) *Biochemistry 18*, 1344–1352.
Malygin, A. G., & Shemyakin, A. G. (1979) *FEBS Lett. 102*, 51–54.
Nath, K., & Hurwitz, J. (1974) *J. Biol. Chem. 249*, 6675–6683.
Neumann, H., & Smith, R. A. (1967) *Arch. Biochem. Biophys. 122*, 354–361.
Nierman, W. C., & Chamberlin, M. J. (1979) *J. Biol. Chem. 254*, 7921–7926.
Schleif, R. F., & Wensink, P. C. (1981) in *Practical Methods in Molecular Biology*, Chapter 5, Springer-Verlag, New York.
Sedmak, J. J., & Grossberg, S. E. (1977) *Anal. Biochem. 79*, 544–552.
Simpson, R. B. (1979) *Cell (Cambridge, Mass.) 18*, 277–285.
Sverdlov, E. D., Tsarev, S. A., & Begar, V. A. (1980) *FEBS Lett. 114*, 111–114.
Travers, A. A., & Burgess, R. R. (1969) *Nature (London) 222*, 537–540.
Wu, C. W., Wu, F. V. H., & Speckhard, D. C. (1977) *Biochemistry 16*, 5449–5454.
Wu, G. J., & Bruening, G. E. (1971) *Virology 46*, 596–612.
Yarbrough, L. R., Schlageck, J. G., & Baughman, M. (1979) *J. Biol. Chem. 254*, 12069–12073.