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

Leslie H. DeRiemer and Claude F. Meares*

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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phosphates (400 Ci/mmol) were from Amersham. Poly[d-(A-T)] was from P-L Biochemicals, Inc. Aquasol scintillation cocktail was from New England Nuclear. Bio-Gel P-30 resin was from Bio-Rad. Kodak type XR X-ray film was used for autoradiograms. Acrylamide (Eastman Kodak) was recrystallized from chloroform. The photoaffinity probes, nucleotides, and poly[d(A-T)] were prepared and purified as described in the preceding paper (DeRiemer & Meares, 1981). During all manipulations prior to photolysis, the photoaffinity probes were handled under reduced light (a 7.5-W bulb) or in total darkness.

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; (G) 0.13 M Tris-HCl, pH 7.9, 16.7 mM $MgCl_2$, 16.7 mM NaCl, 8.1 mM β-mercaptoethanol, 5.8% (v/v) glycerol, and 1.67 mM K_2HPO_4; (H) 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; (I) 89 mM Tris-HCl, pH 7.9, 11 mM $MgCl_2$, 11 mM NaCl, 5.6 mM β-mercaptoethanol, 1.1 mM K_2HPO_4, and 5.6% (v/v) glycerol; (J) 0.1 M Tris-HCl, pH 7.9, 12.5 mM $MgCl_2$, 12.5 mM NaCl, 6.25 mM β-mercaptoethanol, 1.25 mM K_2HPO_4, and 6.25% (v/v) glycerol; (K) 128 mM Tris-HCl, pH 7.9, 20 mM $MgCl_2$, 20 mM NaCl, 2.8 mM β-mercaptoethanol, and 2 mM K_2HPO_4.

Methods

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 the recent modifications (Lowe et al., 1979). 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); bovine serum albumin was used as a standard. A stock solution containing 6.48 μg/μL RNA polymerase in buffer F was used throughout the study unless noted otherwise. Pure RNA polymerase holoenzyme was isolated by chromatography on DNA–agarose; core enzyme was isolated from Bio-Rex 70 (Lowe et al., 1979). Both were stored in buffer F. A solution containing 0.13 mg of core enzyme was concentrated by applying it to a Sephadex A-25 column (0.5 × 1.0 cm), washing the column with 1 column volume of buffer F, and eluting the enzyme with buffer F + 0.5 M NaCl. The core enzyme was then dialyzed into buffer F.

Photoaffinity Labeling RNA Polymerase with a Trinucleotide Probe. In a total volume of 50 μL, the following were combined in the order given: 30 μL of buffer G, 5 μL of poly[d(A-T)] (0.85 μg/μL), 5 μL of N_3PhppApU (1.0 mM), 5 μL of ATP (0.1 mM, 10 μCi/μL [α-^{32}P]ATP) and 5 μL of *E. coli* RNA polymerase (2 μg/μL). The 2 μg/μL RNA polymerase solution was prepared immediately before use by dilution of the 6.48 μg/μL stock solution with H_2O. The above reaction mixture was divided into two 24-μL aliquots and incubated in the dark at 37 °C for 5 min. One sample was photolyzed as described below; the other was kept in the dark for 2 min. Next, 10 μL of buffer H and 1 μL of 1 M dithiothreitol (DTT) were added to each vial. The resulting solutions were allowed to stand in the dark at room temperature for 1.5 h; 64 μL of mock *E. coli* sample (0.6 mL of buffer G, 0.4 mL of H_2O, and 0.4 mL of buffer H) was then added to each sample and, after thorough mixing, three 30-μL aliquots of each sample were each applied to individual wells in the polyacrylamide slab gel described below. RNA polymerase subunits were separated by electrophoresis, and subunit labeling was determined as described below. The procedure is outlined in Figure 1.

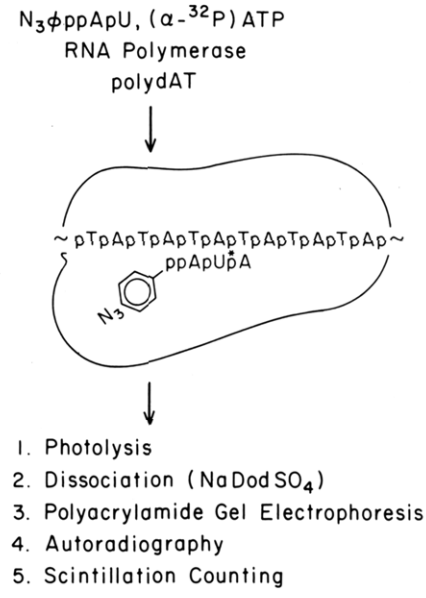

FIGURE 1: Trinucleotide photoaffinity labeling experiment. The radioactive photoaffinity label N_3PhppApUp*A (where the asterisk denotes the position of the ^{32}P label) is synthesized in situ by RNA polymerase. Because the ^{32}P label is only in the 3′ end of RNA, radiolabeling of RNA polymerase subunits is observed only in those ternary complexes which contain trinucleotide RNA. (φ represents Ph = C_6H_4).

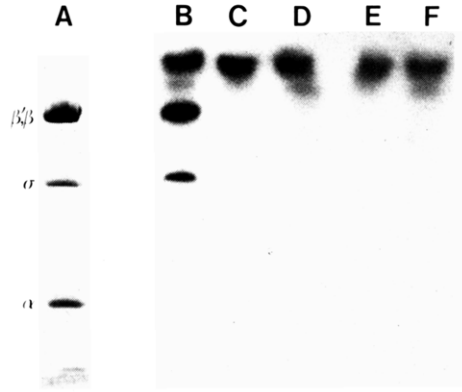

FIGURE 2: Autoradiogram of a trinucleotide photoaffinity labeling experiment and controls. (A) stained gel showing the position of the RNA polymerase subunits. (B) Trinucleotide photoaffinity labeling experiment done as described under Methods. (C) Trinucleotide experiment, missing the photolysis step. (D) Trinucleotide experiment done with 5 μL of buffer E instead of poly[d(A-T)]. (E) Trinucleotide experiment done with 5 μL of H_2O instead of N_3PhppApU. (F) Trinucleotide experiment done with 5 μL of diluted buffer F (15.4 μL of buffer F diluted to 50 μL with H_2O) instead of RNA polymerase. Spots at the top of channels B–F are due to a polymeric impurity in the [α-^{32}P]ATP.

Control reactions, each lacking one feature of the complete procedure, are described in Figure 2. Each of these control reactions had a total volume of 50 μL; after incubation at 37 °C for 5 min and photolysis, 20 μL of buffer H and 1 μL of 1 M DTT were added to each control reaction. After standing in the dark at room temperature for 1.3 h, each was applied in toto to a well in a slab gel. Samples were electrophoresed through a short slab gel and then stained, destained, and autoradiographed as described below.

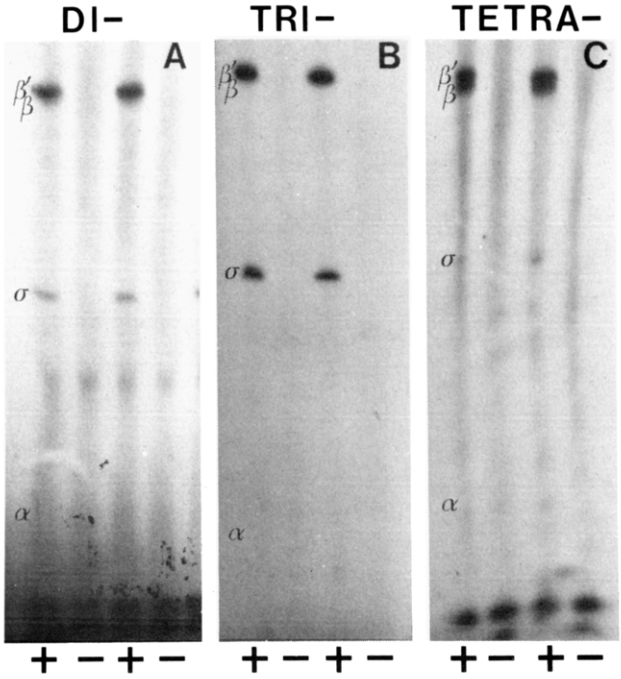

FIGURE 3: Autoradiograms of the polyacrylamide gels of the di-, tri-, and tetranucleotide photoaffinity labeling experiments. Duplicate experiments are shown for each RNA length. + designates a photolyzed experiment and − designates a nonphotolyzed control. The experiments were done as described under Methods. (A) Dinucleotide, (B) trinucleotide, and (C) tetranucleotide autoradiograms.

Another control, comparing labeling on core and holoenzyme, was also done. Six reaction samples were prepared for both core and holoenzyme. Each sample was prepared by combining the following, in the order given, to give a total volume of 45 μL: 25 μL of buffer K, 5 μL of poly[d(A-T)] (0.75 mg/mL), 5 μL of N_3PhppApU (1.0 mM), 5 μL of ATP (0.1 mM, 1 μCi/μL [α-^{32}P]ATP), and 5 μL of RNA polymerase (0.65 μM core or holoenzyme in buffer F). Molecular weights of 457 000 and 375 000 were used for holoenzyme and core enzyme respectively (Lowe et al., 1979). Half of the samples for both core and holoenzyme were photolyzed and the other half were kept in the dark; 20 μL of buffer H and 1 μL of 1 M DTT were then added to each sample, and the samples were kept in the dark at room temperature for 1.5 h. Electrophoresis on short slab gels and quantitation of radiolabeling were as described below.

Photolysis. Solutions containing the ternary complexes were photolyzed in 6 × 20 mm borosilicate glass tubes, which blocked UV radiation below 290 nm. A Rayonet type RS photochemical reactor with six 300-nm UV lamps (type RUL 3000 Å, Southern New England Ultraviolet Co.) was used as a light source. Samples were placed in a rack which held them 8 cm from the lamps and rotated during photolysis. A 2-min photolysis was used throughout; it was sufficiently long to photolyze most of the azide, but short enough to prevent photoinduced cross-linking of the enzyme subunits.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was done by using the $NaDodSO_4$–urea gel system of Wu & Bruening (1971) as modified by Halling et al. (1977). *N,N′*-Diallyltartardiamide was substituted mole-for-mole for methylenebis(acrylamide) to give dissolvable gels (Anker, 1970). Thin (0.75 mm) slab gels were used throughout. Short (10 cm, 7% acrylamide) gels were run for 4 h at 80 V; long (30 cm, 4% acrylamide) gels were run overnight (14 h) at 130 V. The long gels were necessary to separate the β′ and β subunits of *E. coli* RNA polymerase. The distance between the β′ and β peaks was typically 4 mm. Gels were stained for 15 min in 0.25% (w/v) Coomassie Brilliant Blue R-250, 15% (v/v) acetic acid, and 50% (v/v) ethanol and destained for 24 h in 7% (v/v) acetic acid and 25% (v/v) ethanol.

Table I: Percent Distribution of Radioactivity on RNA Polymerase Subunits after Photolysis of Ternary Complexes Containing RNA Chains with an Aryl Azide on the 5′ End[a]

RNA polymerase subunit	photoaffinity label: dinucleotide N_3Ph-ppAp*U (%)	trinucleotide N_3Ph-ppApUp*A (%)	tetranucleotide N_3Ph-ppApUpAp*U (%)
β′	71 ± 7	64 ± 2	88 ± 3
β	6 ± 5	2 ± 0.2	7 ± 2
σ	21 ± 6	35 ± 2	4 ± 4
α	2 ± 3	~0	2 ± 1
	$n = 4$	$n = 3$	$n = 5$

[a] The position of the ^{32}P radiolabel is indicated by an asterisk. The number of experiments used to determine the distribution is designated by *n*. Experiments and quantitation of radioactivity were done as described under Methods. The percentage of bound radioactivity on the subunits was calculated for each experiment and then averaged. Errors represent the standard deviation.

Autoradiograms and Scintillation Counting. Autoradiograms were prepared by freezing the destained gel on dry ice and placing Kodak type XR film in contact with the gel. The film was exposed for 1–5 days at −70 °C. Superposition of the autoradiogram on the gel was used to determine which subunits of RNA polymerase had been labeled. Examples are shown in Figures 2 and 3.

Subunit labeling was quantitated by excision of the subunit bands from the gel, dissolution in 2% (w/v) periodic acid (Anker, 1970), neutralization with 0.21 M sodium bicarbonate, and counting, after addition of 9 mL of Aquasol. For background subtraction an identical, but nonphotolyzed, sample was run in parallel with each photolyzed sample (see Figure 3). This procedure was necessary because some end-on addition of ribonucleotides to the poly[d(A-T)] was observed, particularly with [α-^{32}P]UTP (Nath & Hurwitz, 1974).

Photoaffinity Labeling RNA Polymerase with a Tetranucleotide Probe. In a total reaction volume of 100 μL the following were combined in the order given: 60 μL of buffer G, 10 μL of poly[d(A-T)] (0.6 μg/μL), 10 μL of N_3PhppApU (1.0 mM), 10 μL of ATP (0.1 mM), and 10 μL of *E. coli* RNA polymerase (2 μg/μL). The RNA polymerase solution had been prepared as described for the trinucleotide experiment. The reaction mixture was incubated in the dark at 37 °C for 5 min and then applied to a 0.5 × 7.5 cm Bio-Gel P-30 column. The column was eluted with buffer I. The column effluent was monitored at 254 nm and the ternary complex, which was in the column's excluded volume, was collected. From 10 to 15 min was required to elute the ternary complex from the column. UTP (30 μL, 0.1 mM; 2 or 4 μCi/μL [α-^{32}P]UTP) was immediately added to 270 μL of the effluent containing the ternary complex, and the resulting solution was incubated at 37 °C for 5 min. After incubation, the solution was divided into 48-μL aliquots; half were photolyzed and half were kept in the dark 2 min; 20 μL of buffer H and 1 μL of 1 M DTT were then added to each sample. After sitting 1 h in the dark at room temperature, the samples were each applied in toto to a well in a long slab gel. Electrophoresis, staining, destaining, autoradiography, and quantitation of radioactivity associated with individual subunits were as described earlier. An autoradiogram is shown in Figure 3, and the quantitative results are given in Table I. A control re-

action was prepared as described above except that H_2O was substituted for ATP in the initial incubation.

Photoaffinity Labeling RNA Polymerase with a Dinucleotide Probe. RNA polymerase and poly[d(A-T)] were combined and eluted from a gel exclusion column before addition of N_3PhppA and [α-^{32}P]UTP; 60 μL of buffer G, 20 μL of poly[d(A-T)] (0.85 μg/μL), and 20 μL of *E. coli* RNA polymerase (2 μg/μL) were combined in the order given and eluted from a 0.5 × 7.5 cm Bio-Gel P-30 column with buffer J. The absorbance of the column effluent was monitored at 254 nm and the solution containing the enzyme–DNA complex was collected; 45 μL of N_3PhppA (10 mM) and 45 μL of UTP (0.1 mM, 2 μCi/μL [α-^{32}P]UTP) were immediately added to 360 μL of the effluent solution which contained the enzyme–DNA complex. The mixture was incubated for 5 min at 37 °C and then divided into eight 50-μL aliquots. Half the aliquots were photolyzed and the other half were kept in the dark 2 min; 20 μL of buffer H and 5 μL of 1 M DTT were then added to each aliquot and the mixtures were allowed to stand 1 h at room temperature in the dark. Each aliquot was then applied in toto to a well in a long slab gel. Electrophoresis, staining, destaining, autoradiography, and quantitation of labeling were done as described earlier. An autoradiogram is shown in Figure 3, and the quantitative results are given in Table I.

A control mixture was prepared by eluting RNA polymerase and poly[d(A-T)] from a P-30 column as described above, after which 12.5 μL of H_2O and 12.5 μL of UTP (0.1 mM, 1 μCi/μL [α-^{32}P]UTP) were added to 100 μL of the solution containing the enzyme–DNA complex. The mixture was incubated at 37 °C for 5 min and then divided into two 50-μL aliquots; one aliquot was photolyzed and the other was kept in the dark. The samples were then treated as described above; no subunit labeling was observed.

Results

Photoaffinity Labeling. As shown in Figure 1, formation of the DNA·RNA·RNA polymerase ternary complex was the first step in the trinucleotide photoaffinity labeling experiment. Ternary complexes containing the trinucleotide N_3PhppApUp*A (where the asterisk indicates the location of the ^{32}P label) were generated by the RNA polymerase catalyzed addition of [α-^{32}P]ATP to N_3PhppApU in the presence of a poly[d(A-T)] template. Further polymerization of RNA was prevented by the absence of UTP and UTP derivatives. The ternary complexes were prepared under reduced light to avoid photoreaction of the azide at this stage. For maintenance of enzyme activity, β-mercaptoethanol was used as a reducing agent in all of the buffers. Other reducing agents, such as DTT, have been shown to rapidly reduce aryl azides to amines (Staros et al., 1978). A 2-min photolysis of the ternary complex at 300 nm was followed by dissociation with $NaDodSO_4$ and inactivation of any remaining azide with DTT. After dissociation, the enzyme subunits were separated by polyacrylamide gel electrophoresis and visualized by staining and destaining the gel. The staining and destaining process also served to reduce radioactive background by removing unbound radioactivity from the gel. The final steps of the labeling experiment were locating the enzyme subunits and quantitating the covalently bound label.

For the trinucleotide experiment, labeling takes place only under those conditions which lead to formation of a ternary complex containing a trinucleotide, followed by photoinduced generation of a nitrene. This was shown unequivocally by the results of a series of control experiments in which template, enzyme, N_3PhppApU, or photolysis was omitted. As Figure 2 demonstrates, RNA polymerase subunits were only labeled in the one experiment which contained all of the components needed for ternary complex formation and nitrene generation.

Another control experiment was done to determine if there was any labeling of core enzyme under the conditions of the trinucleotide experiment; no labeling was observed. In a parallel experiment with an equimolar amount of pure holoenzyme, photoaffinity labeling did occur, and the subunit distribution of radioactivity was the same as that observed with a mixture of holoenzyme and core enzyme.

The results of the photoaffinity experiments with the trinucleotide (Figure 3 and Table I) indicate that the 5′ end of a nascent trinucleotide RNA chain is in significant contact only with the β' and σ subunits of *E. coli* RNA polymerase; 64% of the label was associated with the β' subunit and 35% of the radioactivity was associated with the σ subunit. Labeling of the α and β subunits was neither seen on the autoradiograms nor reliably detected by scintillation counting of the gels.

The procedure used to generate a ternary complex containing a tetraribonucleotide photoaffinity label was an extension of the trinucleotide experiment. RNA polymerase, poly[d(A-T)], N_3PhppApU, and nonradioactive ATP were incubated as described for the trinucleotide experiment to generate the ternary complex enzyme·DNA·N_3PhppApUpA. Gel exclusion chromatography on a Bio-Gel P-30 column was used to separate the ternary complex from N_3PhppApU and ATP; this separation was practically quantitative. [α-^{32}P]UTP was added to that fraction of the effluent which contained the ternary complex, and the mixture was incubated at 37 °C for 5 min to form the ternary enzyme·DNA·N_3PhppApUpAp*U complex. Photolysis and analysis of the results were as described for the trinucleotide experiment. An autoradiogram is shown in Figure 3, and the results of several experiments are summarized in Table I. With the tetranucleotide photoaffinity label, the 5′ end of the RNA chain makes contact with β' (88% of the covalently bound radioactivity) and β (7.0% of the covalently bound radioactivity) subunits. Very light labeling of the σ subunit could be seen on the autoradiogram. This band was always visible, but quantitation was difficult. Because it only contained a small fraction of the label, the error limits were of the same magnitude as the amount of labeling. No labeling of the α subunit was observed with the tetranucleotide. Control experiments were also done in which ATP was omitted in order to determine if any labeling due to pyrophosphate-catalyzed exchange of [α-^{32}P]UTP with the ternary complex enzyme·DNA·N_3PhppApU occurred under the experimental conditions used to generate the tetranucleotide (So & Downey, 1970). No labeling was observed.

The dinucleotide photoaffinity experiments were done by combining RNA polymerase and poly[d(A-T)], eluting them from a P-30 column, and adding N_3PhppA and [α-^{32}P]UTP to form the ternary enzyme·poly[d(A-T)]·N_3PhppAp*U complex. When RNA polymerase was added directly to a solution containing N_3PhppA, [α-^{32}P]UTP, and poly[d(A-T)], radioactive products were formed which caused high background radioactivity throughout the length of the polyacrylamide gel. The presence of this background radioactivity, which was possibly poly(uridylic acid), obscured the results of the photoaffinity labeling experiment. Preincubation of the enzyme with template as described above was effective in eliminating the problem. In addition to the nonphotolyzed control experiment, a control experiment was done in which N_3PhppA was omitted; no labeling was observed.

Typical results of the photolysis experiments with the dinucleotide photoaffinity label are shown in Figure 3 and

summarized in Table I. As with the tri- and tetranucleotide photoaffinity labeling experiments, no labeling of the α subunits was observed. The 5′ end of the dinucleotide appears to make contact primarily with the β' (71% of the covalently bound radioactivity) and σ (21% of the covalently bound radioactivity) subunits of RNA polymerase. The β subunit was only very lightly labeled; the labeling could not be detected by looking at the autoradiogram.

Discussion

Malygin & Shemyakin (1979) have shown that nucleoside 5′-phosphates coupled to groups as large as a flavin or nicotinamide mononucleotide moiety can be incorporated in the 5′ position of a nascent RNA chain by RNA polymerase. We have done inhibition and stimulation studies which demonstrate that N_3PhppApU is even better at initiating RNA synthesis than ApU (DeRiemer & Meares, 1981). Of course, the possibility that the probe group causes some minor perturbation of the system cannot be eliminated.

The photoaffinity experiments were designed so that unambiguous labeling results could be obtained. Because the photoaffinity probes used were not substrates for RNA elongation, it was possible to ensure that each labeling experiment was done with a nascent RNA chain of defined length. Incorporation of the radiolabel solely in the 3′ end of the RNA chain also made certain that subunit radiolabeling only occurred in those ternary complexes which contained RNA of the desired length. The use of a photoaffinity label, as opposed to an ordinary reactive group, in the 5′ end of the RNA enabled us to "switch on" the reagent only after the ternary complex containing nascent RNA had been formed.

To be labeled, an RNA polymerase subunit must have been contacted by the photogenerated nitrene. Because nitrenes are fairly nonselective reagents (Knowles, 1971), the absence of labeling suggests that the nitrene and the unlabeled subunit were not in contact. Pseudophotoaffinity labeling, which arises from diffusion of the reactive nitrene away from the desired labeling site (Ruoho et al., 1973), is probably not important in the experiments described here; the lifetime of the RNA polymerase·DNA·RNA ternary complex is several orders of magnitude longer than the 10^{-4}-s nitrene lifetime (Rhodes & Chamberlin, 1974; Knowles, 1971). The fact that the α subunits were never labeled provides convincing evidence that nonspecific labeling does not occur.

Sverdlov and co-workers (1978, 1979) have recently reported experiments with the same goal as those described here. However, their very interesting work suffers from several technical deficiencies which are avoided by our experimental design. As photoaffinity labels, they use mononucleoside triphosphates coupled to *p*-azidoaniline via a phosphoramidate linkage; these compounds are easier to prepare than dinucleotides, but they are very poor initiators of RNA synthesis, also act as weak substrates for *elongation* of RNA, and are unstable to gel staining and destaining procedures. In their experimental design, radiolabeled nucleotides were incorporated throughout the RNA chain rather than only at the 3′ terminus; thus their radiolabeling results come from a *mixture* of oligonucleotides rather than a single chain of defined length. This is implied by the fact that their experiments show the σ subunit still being heavily labeled in a "tetranucleotide" photoaffinity experiment (Sverdlov et al., 1978, 1979). Their use of natural DNA templates containing more than one promoter region, leading to production of different RNAs having similar sequences, is another source of ambiguity. The photoaffinity labels prepared and used in our work are not substrates for elongation and are stable to gel staining and destaining conditions; the dinucleotide N_3PhppApU is a very efficient initiator of RNA synthesis (DeRiemer & Meares, 1981). Radiolabeling only at the 3′ end of nascent RNA provides experimental results which reflect only the surroundings of a single length of RNA in the system we used.

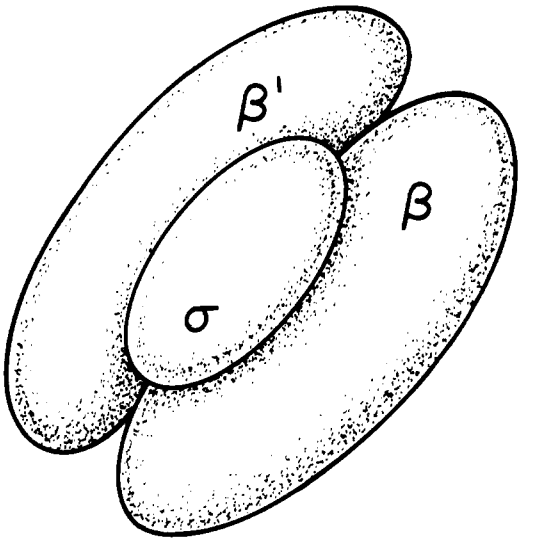

FIGURE 4: Model proposed for *E. coli* RNA polymerase structure, after Hillel & Wu (1977). The results of our photoaffinity labeling experiments and the affinity labeling experiments of Armstrong & Eckstein (1979) show that the elongating RNA is at the interface between the β' and σ subunits. Some labeling of the β subunit was observed with our tetranucleotide RNA photoaffinity probe and with a 3′ affinity label (Armstrong & Eckstein, 1979). Labeling of the α subunits with RNA affinity labels has not yet been observed, so they are not shown.

On the basis of cross-linking studies, Hillel & Wu (1977) have proposed a model for the structure of RNA polymerase (Figure 4) which is also compatible with the results of several different affinity labeling experiments. The β' subunit was the most heavily labeled subunit with our di-, tri-, and tetranucleotide RNA photoaffinity probes; Armstrong & Eckstein (1979) also observed heavy labeling of β' in core enzyme with a series of conventional affinity labels incorporated in the 3′ end of nascent RNA. Thus, it appears that β' contains the principal binding sites for the early RNA product. The results of the di- and trinucleotide photoaffinity experiments also show that RNA is in contact with the σ subunit; therefore, the 5′ end of di- and trinucleotide RNA is either at or near the interface of the β' and σ subunits. Sverdlov et al. (1980) have reported labeling of the σ subunit with a nascent RNA containing a 5-iodouridine residue one position removed from the 3′ end. In addition to making contact with the RNA product, σ has also been shown to be in contact with at least one strand of the DNA template (Simpson, 1979); photolysis of an "open" binary complex of RNA polymerase and a DNA fragment containing 5-bromouridine residues caused labeling of the σ subunit (with the +3 residue) and the β subunit (with the −3 residue). These results and our results with the tetranucleotide photoaffinity probe are consistent with the arrangement of subunits shown in Figure 4. Taken together, our results and those of the other investigators suggest that the catalytic center of RNA polymerase is near the region of the RNA polymerase molecule where the β', β, and σ subunits are in contact with one another.

The most striking result of our photoaffinity labeling experiments is the dramatic change in subunit labeling pattern between a tri- and a tetranucleotide. One attractive explanation for this is that the σ subunit has been released from most of the complexes upon formation of the tetranucleotide.

It is known that the σ subunit can dissociate from the enzyme·DNA·RNA complex during elongation of RNA, and experiments are currently in progress to determine whether or not σ is still associated with the ternary complex in the presence of the tetranucleotide N_3PhppApUpApU.

Another possibility is that the 5′ end of the nascent RNA chain has moved past the σ subunit in going from a tri- to a tetranucleotide. This seems less likely for the following reasons. The distance translocated by the 5′ end of the RNA chain on addition of one nucleotide will depend upon the structure of the complexed RNA; the minimum possible distance is probably 0.26 nm, characteristic of the A-form helix (Beabealashvily et al., 1972; Milman et al., 1967), and the maximum distance translocated could be as large as 0.6 nm if the nascent RNA is stretched out. The fact that the enzyme can catalyze synthesis of RNA bearing any of several quite bulky 5′ substituent groups implies that there is sufficient room for the nitrene photoaffinity reagent to "wobble" in the enzyme·DNA·RNA complex. The nitrene and the 5′ terminal phosphate group are para substituents on a benzene ring and thus are separated by about 0.7 nm. Even relatively restricted motion about the terminal P–O and O–C bonds would therefore make it unlikely that adding a single nucleotide to the nascent RNA chain would change the subunit labeling pattern in the abrupt fashion observed, unless the arrangement of subunits also changes.

A third possible explanation for the large reduction of σ labeling may be that a product-induced conformational change occurs in the enzyme between the tri- and tetranucleotide stages, causing relative movement of the subunits and the product binding site. This possibility may be addressed by other, spectroscopic experiments. For example, by replacing the photoaffinity reagent with a fluorescent energy-transfer probe it should be possible to distinguish between conformational changes and release of the σ subunit.

The large difference between labeling results with the trinucleotide and the tetranucleotide would appear to discount the possibility that nascent RNA can easily move back and forth in the product site of RNA polymerase (McClure, 1980). However, the small amount of labeling of the σ subunit by the tetranucleotide photoaffinity reagent might be due to reversible translocation by the tetranucleotide.

The overall experimental approach described here provides a reliable method for determining the path of nascent RNA across the surface of RNA polymerase. In future experiments we plan to examine longer oligonucleotides, of whatever length needed to define the entire path. Similar experiments using 5′ spectroscopic probes rather than photoaffinity labels are also planned; these may provide complementary information about conformational changes and distances between particular sites on the transcription complex.

Added in Proof

We have confirmed that the trinucleotide and tetranucleotide are produced as described in the text, by using the electrophoresis procedure of Carpousis & Gralla (1980).

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