Identification of a Nucleoside Triphosphate Binding Site on Calf Thymus RNA Polymerase II†

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ABSTRACT: A nucleoside triphosphate binding site on calf thymus RNA polymerase II was identified by using photoaffinity analogues of adenosine 5′-triphosphate and guanosine 5′-triphosphate. Both radiolabeled 8-azidoadenosine 5′-triphosphate (8-N_3ATP) and radiolabeled 8-azidoguanosine 5′-triphosphate (8-N_3GTP) bound to a single polypeptide of this enzyme. This polypeptide has a molecular mass of 37 kilodaltons and an isoelectric point of 5.4. Ultraviolet (UV) irradiation was necessary for photolabeling to occur. In addition, no labeling occurred when the probe was prephotolyzed or when the enzyme was inactivated. Furthermore, photolabeling of the enzyme could be decreased by preincubation with natural substrates. To provide evidence that the radiolabeled polypeptide forms a part of the domain of the nucleoside triphosphate binding site, experiments were performed using unlabeled 8-N_3ATP. Although this unlabeled analogue was not a substrate for RNA polymerase II, it photoinactivated the enzyme in the presence of UV irradiation, and it inhibited transcription elongation by the enzyme in a competitive manner in the absence of UV irradiation. As in the case with photolabeling, photoinactivation by 8-N_3ATP could be decreased by natural substrates; in both cases, purine ribonucleoside triphosphates were more efficient than pyrimidine nucleoside triphosphates. Furthermore, photoinactivation was saturable at about the same concentration as the inhibition constant for 8-N_3ATP. Collectively, these results provide evidence that the radiolabeled polypeptide in calf thymus RNA polymerase II is an essential component for activity and suggest that this polypeptide may be part of this enzyme's purine ribonucleoside triphosphate binding site.

DNA-dependent RNA polymerases are complex enzymes containing many polypeptides. Although an understanding of the general features of the catalytic properties of these enzymes has benefited from a variety of genetic (Greenleaf, 1983), biochemical (Brodner & Wieland, 1976; Cho & Kimball, 1982), and immunological approaches (Breant et al., 1983; Young & Davis, 1983; Tsai et al., 1984; Guilfoyle et al., 1984a,b), little is known regarding which of the polypeptides that copurify with these complex enzymes are required for catalytic activity. This is in part due to the inability to perform reconstitution experiments with eukaryotic RNA polymerases (Burgess, 1976; Lewis & Burgess, 1982; Guilfoyle et al., 1983; Robbins et al., 1984). Thus, except for the likely involvement of the two largest polypeptides of RNA polymerase II in binding α-amanitin (Brodner & Wieland, 1976; Greenleaf, 1983), the subunit composition required for the catalytic activity corresponding to the prokaryotic core enzyme is unknown.

Other attempts to assign a function to each polypeptide in eukaryotic RNA polymerases have involved either cross-linking of ternary complexes by ultraviolet (UV)[1] irradiation (Gundelfinger, 1983) or studies on substrate binding sites using either the nonanalogue pyridoxal 5′-phosphate (Valenzuela et al., 1978) or affinity analogues of substrates, including 9-β-D-arabinofuranosyl-6-mercaptopurine (Cho & Kimball, 1982) and purine nucleoside dialdehydes (Cho et al., 1982). Unlike these chemical affinity probes, limited to binding sites in which only specific functional groups on reactive amino acids are available, photoaffinity probes do not suffer from selective reactivity but will bind to any group present (Plapp, 1983), either in the solvent or in the macromolecule (Bayley & Knowles, 1977).

For example, both 8-N_3ATP and 8-N_3GTP have been used to elucidate the role of many polypeptides in complex enzymes, including bacterial F_1-ATPase (Scheurich et al., 1978), rabbit muscle cAMP-dependent protein kinase (Hoppe & Freist, 1979), sheep brain tubulin (Gaehlen & Haley, 1979), rabbit skeletal muscle phosphorylase kinase (King et al., 1982), calf thymus terminal deoxynucleotidyltransferase (Abraham et al., 1983), bovine F_1-ATPase (Hollemans et al., 1983), *Escherichia coli* DNA polymerase (Abraham & Modak, 1984), rat liver fructose-6-phosphate 2-kinase (Sakakibara et al., 1984), *Chlamydomonas* dynein ATPase (Pfister et al., 1984), and *E. coli* RNA polymerase (Woody et al., 1984).

We report here the results of experiments on the structure of calf thymus RNA polymerase II using 8-N_3ATP/GTP as photoprobes.

Materials and Methods

Chemicals. ^{3}H-Labeled ATP and UTP (specific activities of 56.5 and 43.5 Ci/mmol, respectively) were purchased from New England Nuclear; γ-^{32}P-labeled 8-N_3ATP and 8-N_3GTP (specific activities of 18.8–81 and 13–34 Ci/mmol, respectively) were from ICN. Unlabeled 8-N_3GTP and 8-N_3ATP were gifts from Dr. B. Haley and Dr. A. Kemp, respectively. Poly(dT), calf thymus DNA, ApA dinucleotide, and unlabeled ribonucleoside triphosphates were obtained from Sigma Chemical Co. or Pharmacia P-L Biochemicals.

Characterization of the Enzyme. Calf thymus RNA polymerase II was purified according to the method of Hodo & Blatti (1977). Approximately 6 mg of RNA polymerase II, free of RNA polymerases I and III, was obtained from 1 kg of calf thymus.

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[1] Abbreviations: 8-N_3ATP, 8-azidoadenosine 5′-triphosphate; 8-N_3GTP, 8-azidoguanosine 5′-triphosphate; kDa, kilodalton(s); UV, ultraviolet; K_m^{app}, apparent Michaelis constant; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Activity was assayed according to Hodo & Blatti (1977) in a standard reaction mixture of 100 μL containing 100 mM ammonium sulfate, 2 mM $MnCl_2$, 0.6 mM each of ATP, CTP, and GTP, 5 μg of denatured calf thymus DNA, and 50 mM Tris-HCl (pH 7.9). When a substrate-limiting assay was used, 0.4 μM [^{3}H]UTP (1.25 μCi) was added. When a nonlimiting assay was used, 1.25 μCi of radiolabeled UTP was supplemented with unlabeled UTP to 0.06 mM. Enzyme was added last to initiate the reaction. DNA was omitted to test for endogenous template in the RNA polymerase II fraction. The enzyme fraction was heat-denatured or the template omitted to obtain the background. The incubation was at 37 °C for 10 min. The reaction was stopped by application of the mixture to a GF/C filter (Whatman) and immersion of the filter into 10% (w/w) trichloroacetic acid containing 0.02 mM sodium pyrophosphate. The filters were washed 6 times and dried in 95% (v/v) ethanol and anhydrous ether. After the filters were dried, they were immersed in Econofluor (New England Nuclear), and the radioactivity was counted by liquid scintillation. One unit of enzyme activity is defined as the amount of enzyme which incorporated 1 nmol of UMP per 10 min at 37 °C in a [^{3}H]UMP incorporation assay under near-saturating conditions (Hodo & Blatti, 1977). Typically, the purified enzyme had a specific activity of greater than 200 units/mg of protein. When AMP incorporation was studied, 1.25 μCi of [^{3}H]ATP, diluted to a final concentration of 0.1 mM, was used instead of radiolabeled UTP.

The apparent Michaelis constant (K_m^{app}) for UTP was 19 μM, with a rate constant of product formation of 0.44 s^{-1}, which indicates a turnover number of 1.8 nucleoside monophosphates incorporated per second per RNA polymerase II molecule. The K_m^{app} for ATP was 136 μM, with a rate constant and turnover number of 0.65 s^{-1} and 2.6 nucleoside monophosphates per second per RNA polymerase molecule, respectively.

Studies with a radiolabeled α-amanitin analogue, [^{3}H]-dehydroxymethylamanitylazobenzoyl-*N*-glycylglycine (Preston et al., 1981), demonstrated a dissociation constant of 3×10^{-9} M, an inhibition constant of 6.1×10^{-9} M, and, from Scatchard analyses, a stoichiometric constant of 0.92. Nondenaturing polyacrylamide gel analyses (Maizel, 1971) followed by densitometric scanning of stained gels showed that 95% of the protein migrated as a homogeneous band. Following one-dimensional sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (Laemmli, 1970) and two-dimensional isoelectric focusing/SDS–polyacrylamide gel electrophoresis (O'Farrell, 1975), polypeptides B through L had molecular masses of 180, 143, 43, 36, 25, 20, 18, 17.5, 15.1, 12.1, and 11.0 kDa, respectively, isoelectric points of 8.5, 8.5, 6.0, 5.4, 6.1, 5.8, 5.5, 5.4, 5.0, 5.0, and 5.8, respectively, and stoichiometries of 1.0, 1.0, 0.4, 1.5, 1.6, 1.2, 0.7, 0.3, 2.3, 0.6, and 2.0, respectively, which are consistent with results reported previously (Hodo & Blatti, 1977; Benson et al., 1978).

Inhibition Kinetics. Inhibition kinetics studies used two solutions preincubated separately at 37 °C for 5 min. Solution I contained calf thymus RNA polymerase II, poly(dT), and ApA in 27 μL. Solution II contained ammonium sulfate, $MnCl_2$, 1 μCi of [^{3}H]ATP, unlabeled ATP, Tris-HCl (pH 7.9), and 8-N_3ATP in 23 μL. The final concentrations of the reactants when mixed were 0.12 μM enzyme, 0.3 μM poly(dT), 0.3 mM ApA, 100 mM ammonium sulfate, 2 mM $MnCl_2$, 50 mM Tris-HCl, and ATP concentrations as indicated. The reactions were terminated after 75 s by the addition of 40 μL of 20 mM ethylenediaminetetraacetic acid and 5% (w/w) trichloroacetic acid. The amount of [^{3}H]ATP incorporated into product RNA was determined by liquid scintillation.

Photolysis. Both the radiolabeled and unlabeled photoaffinity compounds were regularly tested by spectrophotometric analysis and thin-layer chromatography to quantify the amount of degradation that had occurred (Potter & Haley, 1983). The average useful life of the radiolabeled probes was 2 weeks at −20 °C. The nonradiolabeled probes were stable for up to a year at −20 °C.

The indicated amounts of 8-N_3-[γ-^{32}P]ATP/GTP were added to 1.5-mL microcentrifuge tubes, and the methanol solvent was removed by a stream of nitrogen. The photolabeling occurred in a solution with a volume of 30–100 μL containing RNA polymerase II, 50 mM Tris-HCl (7.9), 4 mM $MnCl_2$ or $MgCl_2$, and dithiothreitol (DTT) in a concentration range of 0–0.3 mM.

Preincubation took place for 5 min at room temperature in total darkness. The conditions for the cross-linking reaction were exposure of the preincubated mixture at 4 °C for 20 min to UV illumination, with a distance of 2 cm from the filter surface to the microcentrifuge tube. Tubes were rotated every 5 min to ensure equal sample exposure to UV light. The UV source was a type UVSL-25 mineral light (UV Products, Inc.) used in the long-wavelength mode to avoid damage to protein (Koberstein et al., 1976). Control experiments with this source, measuring photolysis of the photoprobes by the change in their absorption spectra, demonstrated half-lives for 8-N_3ATP and 8-N_3GTP of 5–6 min, using a 65 μM concentration at pH 7.0. The photoaffinity labeling reaction was terminated by the addition of DTT and SDS to final concentrations of 25 mM and 1% (w/w), respectively.

Analysis of the photoaffinity-labeled RNA polymerase II enzyme took place by one-dimensional or two-dimensional isoelectric focusing/SDS–polyacrylamide gel electrophoresis, followed by autoradiography of the dried gels. In general, photoproducts derived from a UV-irradiated mixture containing 6 pmol of enzyme and 1 μCi of 8-N_3-[γ-^{32}P]ATP or 8-N_3-[γ-^{32}P]GTP became visible on X-ray film (Cronex 4, Du Pont) within 1 day of exposure.

In those experiments in which the photolabeling was to be performed in the absence of DTT, the enzyme was chromatographed at 4 °C on a Sephadex G-50 (Pharmacia) column (1.2 cm diameter × 5.5 cm) equilibrated with 25% (v/v) glycerol, 5 mM $MnCl_2$, 100 mM ammonium sulfate, 0.1 mM ethylenediaminetetraacetic acid, and 50 mM Tris-HCl (pH 7.9).

The results of the photoinactivation protection experiment were based on three independent assays, yielding three values representing calf thymus RNA polymerase II activity when measured under three different conditions. Reaction A was the control value; it represented total RNA polymerase II activity when measured with the standard transcription assay as described above, i.e., using denatured calf thymus DNA as template and either [^{3}H]UTP undiluted or [^{3}H]ATP diluted with unlabeled ATP to a final concentration of 0.1 mM. The final concentrations of the other nucleoside triphosphates were 1.2 mM. Reaction B was the amount of enzyme activity remaining after photoinactivation, and reaction C was the amount of enzyme activity remaining under the conditions of reaction B, but in the presence of one of the natural substrates, i.e., ATP, GTP, CTP, or UTP, during the photoinactivation. The percentage protection ("% *P*") was calculated from the three parameters *A*, *B*, and *C* by using the equation:

$$\% P = 100[(C - B)/(A - B)]$$

The experimental conditions to derive the value for *A* were

as follows: RNA polymerase II was incubated with prephotolyzed 8-N_3ATP, adenosine, and $MnCl_2$ for 5 min at room temperature and exposed to UV light at 4 °C, and the reaction mixture was completed to measure enzyme activity under standard conditions. To measure *B*, the enzyme was incubated with 8-N_3ATP, adenosine, and $MnCl_2$ for 5 min at room temperature and exposed to UV irradiation at 4 °C, and the reaction mixture was completed to measure the remaining enzyme activity under the standard conditions. *C* was determined by mixing the enzyme with 8-N_3ATP, $MnCl_2$, and one nucleoside triphosphate, i.e., either ATP, GTP, UTP, or CTP. The reaction mixture was incubated for 5 min at room temperature and exposed to UV irradiation at 4 °C, and the reaction mixture was completed, including the addition of adenosine, to measure the partial recovery of enzyme activity under standard conditions.

The initial concentration of 8-N_3ATP was 0.55 mM, and the ribonucleoside triphosphate substrate concentration in reaction C was 3 mM during preincubation and UV irradiation. After preincubation of reaction mixtures A, B, and C in the dark in a total volume of 20 μL, the mixture was irradiated with UV light for 40 min. The remaining assay components were added including denatured calf thymus DNA and either 0.6 μCi of [^{3}H]UTP or 0.6 μCi of [^{3}H]ATP in the final reaction volume of 50 μL. After additions were made to complete the reaction mixtures, the final concentration of the ribonucleoside triphosphate was 1.2 mM. Because of this concentration, neither UTP nor ATP could be tested as a protective agent when [^{3}H]UMP or [^{3}H]AMP was used with incorporation assays, respectively, due to excessive isotope dilution. The typical values obtained for reaction A were 110000 and 23000 cpm above background (200 cpm), when measured in the [^{3}H]UMP and [^{3}H]AMP incorporation assays, respectively.

Results

Mode of Inhibition by 8-N_3ATP. Preliminary experiments (not shown) indicated that 8-N_3ATP could not substitute for ATP in a transcription reaction in vitro. The possibility that 8-N_3ATP is an inhibitor of calf thymus RNA polymerase II was investigated in the absence of UV light by using the standard transcription assay with calf thymus DNA and 1.2 μCi of [^{3}H]UTP. Enzyme activity was measured in identical reaction mixtures, each containing 6 pmol of enzyme, to which were added increasing amounts of 8-N_3ATP to the final concentrations indicated in Figure 1. The experiment was repeated with 1.2 μCi of [^{3}H]ATP diluted to a final concentration of 0.1 mM. The results in Figure 1 demonstrate that 2 mM 8-N_3ATP inhibited the enzyme activity by 50% and 80% when [^{3}H]UTP and [^{3}H]ATP were used in the incorporation assay, respectively, and that prephotolyzed 8-N_3ATP did not inhibit the enzyme activity to the same degree as intact 8-N_3ATP.

Since the results of Figure 1 show that 8-N_3ATP was an inhibitor of the transcription reaction by calf thymus RNA polymerase II with respect to [^{3}H]AMP and [^{3}H]UMP incorporation into RNA, it was important to determine whether the inhibition was competitive or noncompetitive. However, the transcription reaction with calf thymus DNA as template is complex and poses several problems in studies on the kinetics of inhibition by a substrate analogue. For example, the enzyme requires four different substrates in amounts and in a sequence determined by the heterogeneous calf thymus template and not by the individual substrate concentrations present during the assay. In addition, the overall rate of incorporation, as determined by measuring the incorporation of [^{3}H]AMP, for example, is composed of two separate events that occur during the transcription assay. These events, initiation and elongation, are known to occur at different rates (Kadesch & Chamberlin, 1982; von Hippel et al., 1984). Initiation is the rate-limiting step because of the time needed to form a ternary complex, composed of RNA polymerase template and two nucleotides, and to form the first phosphodiester bond. Furthermore, once the enzyme has dissociated from the template, reinitiation can take place, causing another lag in the transcription rate.

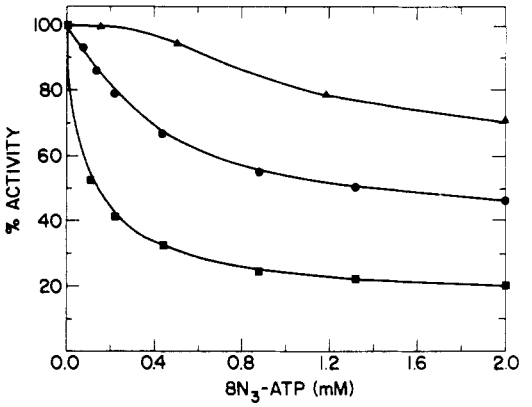

FIGURE 1: Noncovalent inhibition of calf thymus RNA polymerase II activity by 8-N_3ATP. Standard transcription assays with calf thymus DNA as template, each containing 6 pmol of calf thymus RNA polymerase II, were titrated with increasing concentrations of azido-ATP. The enzyme activity was measured in the presence of tritiated UTP (●) or tritiated ATP (■) as described under Materials and Methods. The effect of the addition of prephotolyzed azido-ATP was also measured with respect to tritiated AMP incorporation (▲).

To facilitate the study of inhibition kinetics here, several experimental modifications were made in an attempt to measure the reaction velocity only during the elongation phase of transcription. First, a synthetic poly(dT) template was used instead of calf thymus DNA, thus limiting the kinetics assay to a single substrate. Second, a preformed ternary complex, composed of poly(dT), calf thymus RNA polymerase II, and ApA, was used to start the reaction. Dinucleotides are efficient initiators of transcription reactions (DeRiemer & Meares, 1981a,b; Wilkinson & Sollner-Webb, 1982; Samuels et al., 1984). Finally, the incubation time was limited to 75 s instead of the usual 10 min in an attempt to limit reinitiation as well as substrate depletion. When all reactants were warmed to 37 °C prior to mixing, no initial lag in enzyme velocity occurred. The results of experiments using these modifications are shown in Figure 2. The K_m^{app} for ATP was 0.77 mM with a turnover number of 2.9 molecules of AMP incorporated per enzyme molecule per second, assuming all enzymes present in the assay participated simultaneously. The inhibition constant of 8-N_3ATP was 0.40 ± 0.04 mM. The pattern shown in the Lineweaver–Burk plot indicates competitive inhibition.

Irreversible Light-Induced Inactivation of Calf Thymus RNA Polymerase II by Azido-ATP. If 8-N_3ATP binds to a domain of calf thymus RNA polymerase II that is part of a nucleoside triphosphate binding site(s), the 8-N_3ATP should inactivate the enzyme irreversibly when the complex is exposed to UV irradiation by reacting with amino acid residues in this (these) binding site(s). To test this, the enzyme was mixed with increasing concentrations of 8-N_3ATP in a standard reaction mixture containing 0.1 mM DTT and exposed to UV irradiation for 90 min. This extended exposure was necessary because of absorption by 8-N_3ATP at the concentrations used. The activity of the enzyme after UV irradiation was assayed immediately and corrected for two types of inhibition not

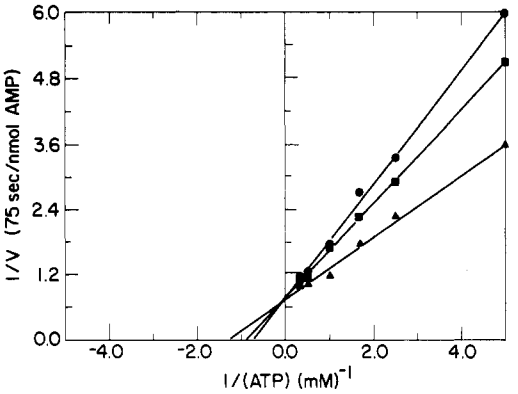

FIGURE 2: Inhibition of RNA synthesis by 8-N_3ATP in the absence of UV irradiation. Three double-reciprocal plots of initial velocity vs. ATP concentration are shown. The concentrations of calf thymus RNA polymerase II, poly(dT) template, ApA dinucleotide, ammonium sulfate, $MnCl_2$, labeled ATP, and Tris-HCl (pH 7.9), as well as the reaction conditions, were as detailed under Materials and Methods. The ATP concentrations used are indicated in the figure. The final concentrations of the inhibitor azido-ATP were 0 (▲), 0.15 (■), and 0.3 mM (●). The reactions were terminated after 75 s by the addition of 40 μL of 20 mM ethylenediaminetetraacetic acid and 5% (w/w) trichloroacetic acid.

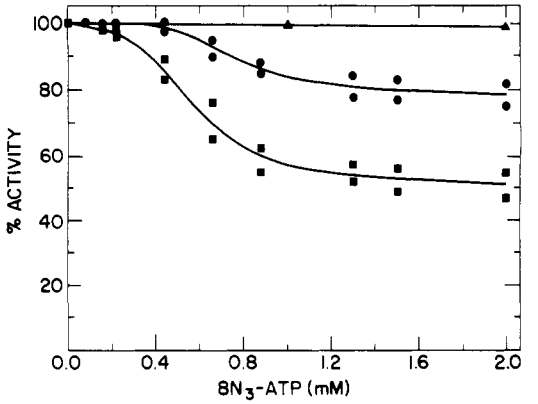

FIGURE 3: Photoinactivation of calf thymus RNA polymerase II by 8-N_3ATP, measured with respect to UMP incorporation. Azido-ATP was added to standard reaction mixtures, containing 3 pmol of calf thymus RNA polymerase II and 4 mM $MnCl_2$, to the final concentrations within the preincubation mixture as indicated. After preincubation, the reaction mixtures were exposed to UV irradiation for 90 min at 4 °C. The activity of the enzyme was assayed immediately, using undiluted tritiated UTP as described under Materials and Methods. After 10 min at 37 °C, the reaction was terminated. The experiment was repeated with prephotolyzed 8-N_3ATP as a control. Shown are the results indicating enzyme activity remaining after incubation and UV irradiation of the enzyme with prephotolyzed 8-N_3ATP (●) and the results obtained with intact 8-N_3ATP, after correction for inhibition by the prephotolyzed azido probe (■). As another control, the experiment described above was repeated by preincubating and UV irradiating the enzyme with ATP at final concentrations as indicated (▲) on the abscissa.

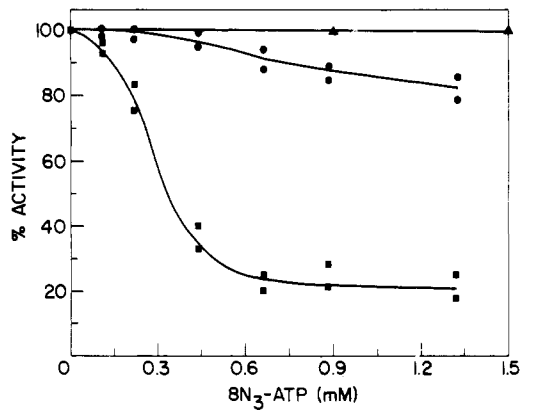

FIGURE 4: Photoinactivation of calf thymus RNA polymerase II by 8-N_3ATP, measured with respect to AMP incorporation. The assay was performed as described in the legend of Figure 3, with the exception that tritiated AMP instead of tritiated UMP was monitored. The 1.2 μCi of labeled ATP was diluted to a final concentration of 0.1 mM. Shown are the results indicating enzyme activity remaining after incubation and UV irradiation of the enzyme with prephotolyzed 8-N_3ATP (●) and intact 8-N_3ATP, after correction for inhibition by the prephotolyzed azido probe (■). As another control, the experiment described above was repeated by preincubating and UV irradiating the enzyme with GTP instead of ATP, since the latter substrate would dilute the labeled ATP too much at the concentrations used (▲).

caused by irreversible photoinactivation. The first inhibition resulted from exposure of the enzyme to UV irradiation and amounted to a loss of 10% of the activity under the conditions used. The remaining 90% of the activity was arbitrarily set at 100% activity. The second noncovalent inhibition was caused by the presence of photolyzed 8-N_3ATP during the enzyme activity assay after UV irradiation (see controls in Figures 1 and 3). The data showing irreversible photoinactivation were obtained by subtracting the percent of enzyme inhibition obtained with prephotolyzed 8-N_3ATP from the percent of inhibition determined by using intact 8-N_3ATP. The resulting values were plotted as a function of the 8-N_3ATP concentrations with respect to [^{3}H]UMP and [^{3}H]AMP incorporation and are shown in Figures 3 and 4, respectively. The photoinactivation by 8-N_3ATP was irreversible because addition of ATP after UV exposure to the 0.6 mM ATP already present in the standard activity assay did not increase the activity of the enzyme. Additional control experiments were performed by preincubating RNA polymerase II with the natural substrates ATP and GTP instead of photolabel at the concentrations indicated for 8-N_3ATP, followed by UV irradiation; no inhibition of enzyme activity was observed. Furthermore, when prephotolyzed 8-N_3ATP was used, no photoinactivation occurred.

Protection by Nucleoside Triphosphates against Photoinactivation of Calf Thymus RNA Polymerase II. True photoaffinity labeling requires that the probe interact with the natural substrate binding site, which in the case of RNA polymerase II is the nucleoside triphosphate binding site. Previous experiments indicated that 8-N_3ATP could irreversibly photoinactivate calf thymus RNA polymerase II and that its mode of inhibition is competitive during the elongation phase of transcription. Therefore, photoinactivation by 8-N_3ATP should be partially prevented by coincubation of RNA polymerase II with the natural substrates.

In the design of the assay, several complications had to be addressed. For example, added nucleoside triphosphates will absorb UV light, thus decreasing the number of photons available for absorption by the photoprobe. This in turn would decrease the rate of photoinactivation merely by lowering the rate of nitrene formation. As a control, adenosine was added to a final concentration calculated to result in an absorbance identical with that for solutions containing nucleoside triphosphates. It was established that no photoinactivation of calf thymus RNA polymerase II was caused by UV irradiation of a mixture containing enzyme and 2.5 mM adenosine. Furthermore, the effect of the added adenosine during standard transcription assays was determined; the inhibition was minor. In addition to the added adenosine, another factor for which correction had to be made was the photolyzed 8-N_3ATP, as detailed under Materials and Methods.

The amount of protection by the nucleoside triphosphates, used individually, was 45 ± 10%, 41 ± 9%, and 12 ± 5% with

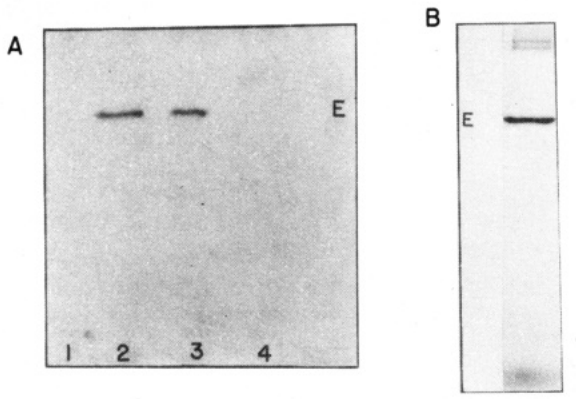

FIGURE 5: Photoaffinity labeling of calf thymus RNA polymerase II with radiolabeled 8-N_3ATP or with radiolabeled 8-N_3GTP. Shown is an autoradiograph of a dried one-dimensional SDS–polyacrylamide gel of calf thymus RNA polymerase II enzyme following photoaffinity labeling. (A) Lane 1, 7 pmol of enzyme was incubated with 2 μCi of radiolabeled 8-N_3ATP and 4 mM $MnCl_2$ in the dark for 30 min at 22 °C; lane 2, same as lane 1, but irradiated with UV light for 15 min at 4 °C; lane 3, same as lane 2, but with radiolabeled 8-N_3GTP; lane 4, same as lane 1, but with radiolabeled 8-N_3GTP. All mixtures contained 0.3 mM DTT. (B) Autoradiogram of the enzyme incubated with radiolabeled 8-N_3ATP in the presence of 4 mM $MnCl_2$ but in the absence of DTT.

ATP, GTP, and CTP, respectively, when the labeled substrate was [^{3}H]UTP. Protection of 25 ± 9%, 5 ± 3%, and 9 ± 3% with GTP, CTP, and UTP, respectively, was observed when the labeled substrate was [^{3}H]ATP.

Photoaffinity Labeling of RNA Polymerase II and Polypeptide Analyses. UV irradiation of a reaction mixture containing calf thymus RNA polymerase II and radiolabeled 8-N_3ATP or radiolabeled 8-N_3GTP induced covalent cross-linking of the radioactive photoprobes to the enzyme, whereas no incorporation of label occurred in the absence of UV irradiation. Of the 11 polymerase polypeptides resolved by one-dimensional SDS–polyacrylamide gel electrophoresis (see Materials and Methods), only a single polypeptide with a molecular mass of 37 kDa was found to be radiolabeled by each azidopurine analogue (Figure 5A). Radiolabeling of a polypeptide of this molecular mass indicates that the azido-radiolabeled target is polypeptide E of the calf thymus RNA polymerase II, according to the nomenclature established previously (Hodo & Blatti, 1977). This pattern of radiolabeling did not change after preincubation of the enzyme before UV irradiation with 4 μg of various templates, including denatured or native calf thymus DNA, poly(dT), or poly(dC), or in the presence of 10 ng of α-amanitin/mL. Furthermore, no photoaffinity labeling occurred when the enzyme was inactivated by heat denaturation (80 °C for 5 min) or when the photoprobes were prephotolyzed by UV irradiation.

The photoaffinity labeling experiments described thus far were done in the presence of the radical scavenger DTT, a component of the enzyme storage buffer at a concentration of 0.3 mM. DTT will, in addition, reduce azides to amines and thereby destroy photoactivity before photolysis. An indication of this can be seen in Figures 3 and 4, where little photoinactivation occurs at photoprobe concentrations below that of the DTT. To determine the effect of this thiol on the radiolabel incorporation pattern seen by one-dimensional SDS–polyacrylamide gel electrophoresis, the experiment described in the legend of Figure 5 (panel A) was repeated in the absence of DTT. The two largest polypeptides present in calf thymus RNA polymerase II (B and C) were then labeled in addition to polypeptide E. However, polypeptide E remained the principal target of the photolabeling. Less than 1% of the label was found associated with polypeptides B and C.

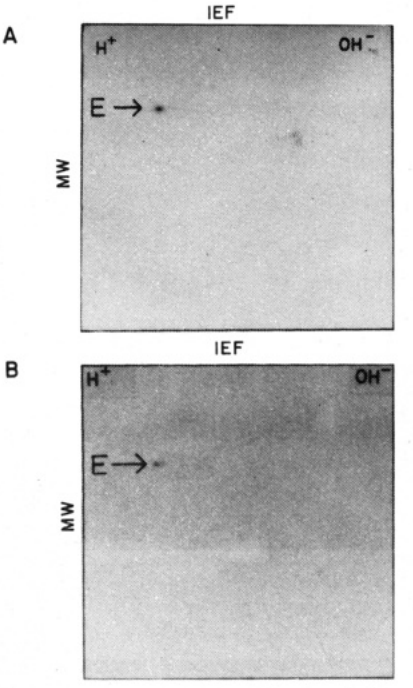

FIGURE 6: Analysis by two-dimensional isoelectric focusing/SDS–polyacrylamide gel electrophoresis of polypeptides from photolabeled calf thymus RNA polymerase II. (A) Calf thymus RNA polymerase II (10 μg, DTT free) was incubated with 1 μCi of radiolabeled 8-N_3ATP and 4 mM $MnCl_2$, UV-irradiated for 30 min at 4 °C, and then subjected to two-dimensional analyses and autoradiography. (B) Calf thymus RNA polymerase II (10 μg, DTT free) was incubated with 3 μCi of radiolabeled 8-N_3GTP and 4 mM $MnCl_2$ and analyzed as described in panel A.

To characterize further the major radiolabeled polypeptide in calf thymus RNA polymerase II, two-dimensional isoelectric focusing/SDS–polyacrylamide gel electrophoresis was performed. The results in Figure 6 demonstrate that the 37-kDa radiolabeled polypeptide has an isoelectric point of 5.2. This is again consistent with the identification of the azido-radiolabeled polypeptide as E (Hodo & Blatti, 1977). In addition to confirming the molecular weight and establishing the isoelectric point of the major radiolabeled polypeptide, the two-dimensional analyses demonstrated a small shift of 0.2 pH unit in the acidic direction of the isoelectric point of polypeptide E after cross-linking of the photoprobes. That is, comparison of the silver-stain pattern of the 37-kDa species, before and after photoaffinity labeling, with the autoradiographic pattern revealed that after radiolabeling, a small amount of new silver-stained material appeared at a position which was 0.2 pH unit in the acidic direction away from the main body of silver-stained protein at an isoelectric point of 5.4 (data not shown). This new spot contained virtually all of the radiolabel. No photolabeling of any of the other polypeptides was observed by two-dimensional analysis after radiolabeling in the absence of DTT, which might be due to prolonged exposure of the ^{32}P of the labeled azido analogue on the two basic polypeptides, B and C, to an alkaline environment during electrophoresis in the first dimension.

Optimization of Conditions for Cross-Linking of Probes to RNA Polymerase II. The efficiency of cross-linking of radiolabeled 8-N_3ATP and radiolabeled 8-N_3GTP to calf thymus RNA polymerase II was determined as a function of increasing time of UV irradiation and is shown in Figure 7. Also shown are the autoradiographs from which the data were derived. The maximal amount of radioactivity incorporated at 30 min was typically 3–4.5 kcpm.

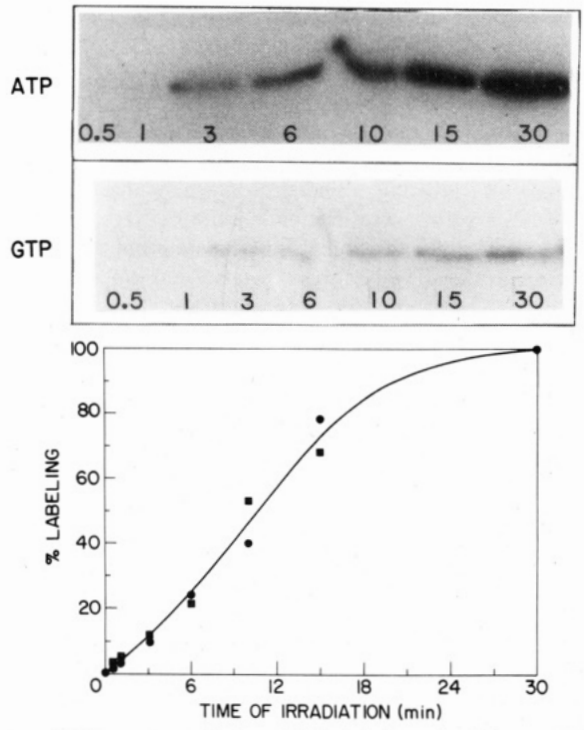

FIGURE 7: Time course of photoaffinity labeling of calf thymus RNA polymerase II by radiolabeled 8-N_3ATP and 8-N_3GTP. A standard reaction mixture of 50 μL contained 10 pmol of calf thymus RNA polymerase II (free of DTT), 4 mM $MnCl_2$, and 1 μCi of either 8-N_3ATP or 8-N_3GTP. After preincubation as described under Materials and Methods, the reaction mixtures were exposed to UV irradiation for the indicated amounts of time. After termination of the reaction, the samples were analyzed by one-dimensional SDS–polyacrylamide gel electrophoresis and stained. After autoradiography, the appropriate bands were excised, and the amount of cross-linked radiolabeled 8-N_3ATP or 8-N_3GTP was determined by liquid scintillation counting. The amount of labeling obtained during different time intervals is expressed as the percent of labeling achieved by irradiation for 30 min. Both the autoradiographs and time course of radiolabel incorporation are shown for the enzyme with radiolabeled 8-N_3ATP (●) and 8-N_3GTP (■). The number under each band indicates the time of UV irradiation in minutes.

Stoichiometric Analysis of Photoaffinity Labeling. The extent of covalent attachment of the radiolabeled photoprobes 8-N_3ATP and 8-N_3GTP to the enzyme was measured by using different amounts of the photoprobes to determine at which concentration the saturation of labeling occurs. The standard reaction mixtures contained enzyme and photoaffinity label which was diluted by adding unlabeled photoprobe to the final concentrations indicated in each figure. The mixtures were preincubated and UV-irradiated, and the extent of cross-linking was determined as described in the legend of Figure 7. The number of picomoles of photoprobe molecules bound per picomole of RNA polymerase II was plotted as a function of total photoprobe concentration. The stoichiometry of 8-N_3ATP and 8-N_3GTP radiolabeling of calf thymus RNA polymerase II is shown in Figure 8. These results demonstrate that between 0.3 and 0.5 mol of azido photoprobe was bound per mole of enzyme.

Protection against Photoaffinity Labeling by Natural Substrates. If the labeling of RNA polymerase II by photoprobes is specific with respect to binding in a domain of the enzyme that is part of the substrate binding site, then the natural substrates would be expected to protect the enzyme from photoaffinity labeling. The degree of protection of calf thymus RNA polymerase II from photoaffinity labeling was determined in the presence of different concentrations of each of the substrate nucleoside triphosphates during preincubation

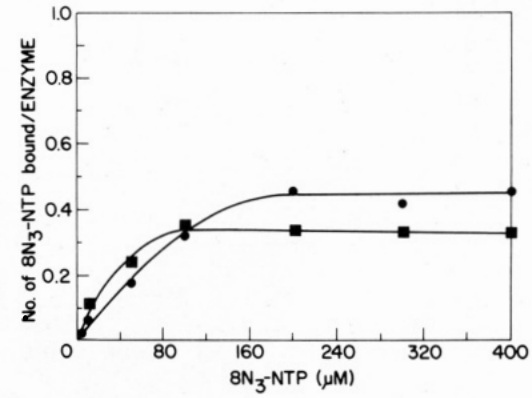

FIGURE 8: Number of radiolabeled 8-N_3NTP molecules bound per calf thymus RNA polymerase II molecule vs. the 8-N_3NTP concentration. The standard reaction mixture contained 10 pmol of calf thymus RNA polymerase II (free of DTT) and 1 μCi of radiolabeled 8-N_3ATP (●) or 8-N_3GTP (■), which was diluted by addition of unlabeled 8-N_3NTP to the final concentrations as indicated. After exposure to UV irradiation for 30 min, the samples were analyzed by one-dimensional SDS–polyacrylamide gel electrophoresis, stained, and autoradiographed, and the amount of cross-linked radiolabeled 8-N_3NTP was quantified as described in the legend of Figure 7. The number of photoprobe molecules covalently cross-linked per molecule of calf thymus RNA polymerase II was plotted as a function of the photoprobe concentration.

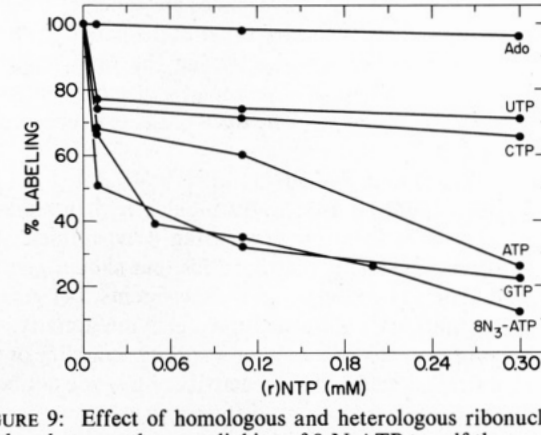

FIGURE 9: Effect of homologous and heterologous ribonucleoside triphosphates on the cross-linking of 8-N_3ATP to calf thymus RNA polymerase II. Standard reaction mixtures of 50 μL contained 10 pmol of calf thymus RNA polymerase II (free of DTT) and 1 μCi of radiolabeled 8-N_3ATP, which was diluted with unlabeled 8-N_3ATP as indicated or to which was added either ATP, GTP, CTP, or UTP, before the preincubation, to the final concentrations indicated. Ado represents control experiments in which adenosine was added to the preincubation mixture in place of the ribonucleoside triphosphate. The reaction mixtures were exposed to UV irradiation as described under Materials and Methods, and the amount of cross-linked radiolabel was determined by counting the radioactivity in band E excised from one-dimensional SDS–polyacrylamide gels.

and UV irradiation. The results were plotted as a function of concentration and are shown in Figures 9 and 10 for the enzyme incubated with radiolabeled 8-N_3ATP and radiolabeled 8-N_3GTP, respectively. As in the case of protection from photoinactivation of the enzyme, the purine substrates offer better protection than the pyrimidine substrates. Absorption of UV irradiation by the added natural substrates is not responsible for the protection, since, in a control experiment, the addition of adenosine to the enzyme and radiolabeled 8-N_3ATP hardly affects incorporation of the photoprobe under the conditions used.

DISCUSSION

Eukaryotic RNA polymerases are complex multi-polypeptide enzymes that transcribe DNA by polymerization of four different triphosphates into complementary RNA prod-

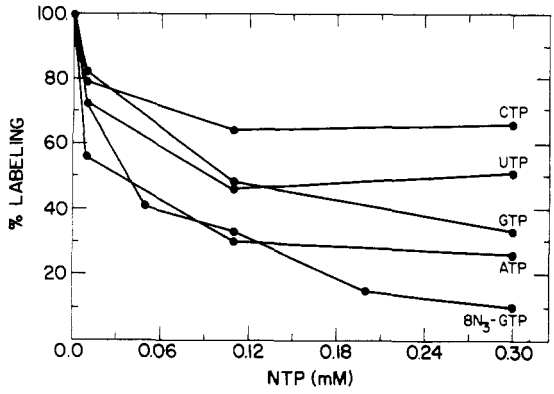

FIGURE 10: Effect of homologous and heterologous ribonucleoside triphosphates on the cross-linking of 8-N_3GTP to calf thymus RNA polymerase II. Standard reaction mixtures of 50 μL contained 10 pmol of calf thymus RNA polymerase II (free of DTT) and 1 μCi of radiolabeled 8-N_3GTP, which was diluted with unlabeled 8-N_3GTP as indicated or to which was added either ATP, GTP, CTP, or UTP, before the preincubation, to the final concentrations indicated. The reaction mixtures were exposed to UV irradiation, and the amount of cross-linked radiolabel was determined.

ucts. The enzyme contains several sites which bind the DNA template, substrates, and the product RNA. The study of the molecular structure of this enzyme should answer three fundamental questions. Which polypeptides are subunits? Which polypeptides are adjacent to each other in the native enzyme? How do these subunits interact during the four stages of transcription, i.e., recognition of promoter sequences, initiation, elongation, and termination? The work presented here focuses on the first question.

Saraste (1983) and Robbins et al. (1984) stated several criteria which could be used to distinguish real subunits in polymeric enzymes from contaminating polypeptides: (1) copurification, (2) stoichiometric ratios that should give integral numbers, (3) reconstitution experiments, (4) genetic mapping of mutants with a defective enzyme activity, (5) localization of sites that bind either a specific inhibitor or the natural substrate, and (6) cross-reactivity of a single antibody with similar polypeptides from different organisms.

Copurification of polypeptides is a weak argument which can be strengthened somewhat by showing integral stoichiometric ratios. However, even this is insufficient for a polypeptide to qualify as a functional subunit, and at least one more criterion should be tested before a polypeptide can be placed among the subunits. The work presented here addresses the fifth criterion. Most research on the localization of true subunits in eukaryotic RNA polymerases has used tests satisfying this criterion, by using the specific inhibitor α-amanitin. These tests have pointed to the two largest subunits in eukaryotic RNA polymerase II as sites for binding this inhibitor (Brodner & Wieland, 1976; Greenleaf, 1983). The research presented here attempts to locate true subunit(s) by determining which polypeptide(s), among the polypeptides that copurify in calf thymus RNA polymerase II, bind(s) nucleoside triphosphates. The conclusions drawn from these experiments should, however, be further corroborated by experiments that test the third and fourth criteria, for which the experimental approaches reported in studies of prokaryotic RNA polymerase are good examples (Saitoh & Ishihama, 1976; Scaife, 1976; Yura & Ishihama, 1979).

The nucleoside triphosphate derivatives used here were the photoaffinity labels 8-N_3-[γ-^{32}P]ATP and 8-N_3-[γ-^{32}P]GTP. These photoprobes are very reactive and do not require the presence of any specific amino acid residue at the active site for covalent incorporation. To show binding site specificity in photoaffinity labeling, the results must satisfy several criteria (Woody et al., 1984). The photoprobe should be either a substrate or a competitive inhibitor of the enzyme. The photoprobe should be able to photoinactivate and photoaffinity label the enzyme, and the natural substrates should at least partially protect the enzyme from both photoinactivation and photoaffinity labeling. The site on which the photoprobe interacts should show saturation kinetics with respect to both photoinactivation and photoaffinity labeling. Finally, control experiments should be performed to show that the photoaffinity labeling is UV irradiation dependent and takes place only under conditions at which both the enzyme and the photoprobe are intact.

The experimental results presented here led to the identification of a single major site of photoaffinity labeling in calf thymus RNA polymerase II by azidopurine nucleoside triphosphate analogues. This site is located on a polypeptide, with a molecular mass of 37 kDa and an isoelectric point of 5.4, known as polypeptide E, according to the nomenclature used to describe the polypeptide compositions of calf thymus RNA polymerase II enzyme (Hodo & Blatti, 1977).

This polypeptide E contains one or more domains that appear to be involved in the binding and/or catalytic stage during transcription. This conclusion is based on several observations. The mode of inhibition by 8-N_3ATP in the absence of UV irradiation was competitive with respect to the incorporation of [^{3}H]AMP during the elongation phase of the transcription. 8-N_3ATP inactivated calf thymus RNA polymerase irreversibly in the presence of UV light. UV irradiation was absolutely necessary for irreversible inactivation and photoaffinity labeling. The enzyme was partially protected from photoaffinity labeling and photoinactivation by the presence of the natural substrate nucleoside triphosphates during both preincubation and UV irradiation. A native conformation of the RNA polymerase II was necessary for photoaffinity labeling. At high concentrations of the photoprobe during UV irradiation, a saturation effect occurred in photoaffinity labeling and photoinactivation. Furthermore, the concentrations of 8-N_3ATP concentrations at which saturation occurred in photoinactivation and photoaffinity labeling are 600 and 200 μM, respectively. These concentrations are consistent with the inhibition constant value of 400 μM, indicating that covalent photoaffinity labeling and covalent photoinactivation may occur at the same site at which noncovalent but competitive inhibition occurs by 8-N_3ATP toward AMP incorporation during the elongation phase of a transcription reaction.

In addition to RNA polymerases, calf thymus contains protein kinases (Kranias et al., 1977; Kranias & Jungmann, 1978a,b; Dahmus, 1971a–c; Gordon et al., 1983; Dahmus et al., 1984). It was of concern, therefore, that the calf thymus RNA polymerase II preparation used here was contaminated by a kinase which could interact with the photoprobes either by binding them at its active site or by transferring the radiolabel to polypeptide E of calf thymus RNA polymerase II. However, several control experiments (not shown) failed to demonstrate the presence of a contaminating protein kinase. For example, if polypeptide E is labeled by a protein kinase using the photoprobe as phosphate donor, then radiolabeling should be independent of the presence of UV irradiation. However, in the absence of UV irradiation, no phosphorylation of any RNA polymerase II polypeptide occurred with either [γ-^{32}P]ATP or 8-N_3-[γ-^{32}P]ATP or 8-N_3-[γ-^{32}P]GTP when analyzed by trichloroacetic acid precipitable counts or denaturing polyacrylamide gel electrophoresis followed by auto-

radiography. Alternatively, the radiolabeled protein could be a protein kinase with a molecular mass and isoelectric point identical with those of calf thymus RNA polymerase II polypeptide E. However, polypeptide E and the most likely contaminating protein kinase from calf thymus, casein kinase I, do not have identical electrophoretic mobilities when run on parallel lanes of a denaturing polyacrylamide gel, nor do they have similar isoelectric points following two-dimensional isoelectric focusing/SDS–polyacrylamide gel analysis; the isoelectric points of polypeptide E and casein kinase are 5.4 and 9, respectively. Finally, there was no cross-reaction of antibody to casein kinase I with calf thymus RNA polymerase II peptide E (M. Dahmus, personal communication). Collectively, these data suggest that the calf thymus RNA polymerase II preparation, purified as described previously (Hodo & Blatti, 1977) and radiolabeled with 8-N_3ATP/GTP photoprobes as described here, contains no kinase contaminant which can account for the specific labeling of a 37-kDa polypeptide with an isoelectric point of 5.4.

The involvement of other domains on different peptides of RNA polymerase II in binding nucleoside triphosphates and/or in the polymerization reaction is by no means excluded, because in the absence of the radical scavenger DTT, some minor labeling of the two largest polypeptides occurred in addition to the labeling of polypeptide E. Of interest, previous reports have implicated the large polypeptides in eukaryotic RNA polymerases as components of the substrate binding site(s) (Valenzuela et al., 1978; Cho & Kimball, 1982; Cho et al., 1982). This apparent inconsistency with the results reported here may reflect the differences between chemical affinity labeling, which requires a reactive site chemically compatible with the analogue, and photoaffinity labeling, which does not. Alternatively, these results may indicate that the 37-kDa polypeptide and the large polypeptides are adjacent in the nucleoside triphosphate binding domain. Indeed, Ruet et al. (1980) proposed a similar association of subunits from genetic analyses of yeast RNA polymerase II.

The selectivity and degree of covalent labeling of RNA polymerase by any nucleoside triphosphate derivative in vitro will depend on many factors, including the efficiency of Watson–Crick base pairing by the particular nucleoside triphosphate analogue with the template strand, and the microenvironment, i.e., the ionic strength, pH, and presence of competing nucleoside triphosphates. In addition, there will be the factor of a varying molecular distance between the highly reactive substrate–nitrene intermediates and the peptide chain domains during all four phases of the transcription reaction. All these factors warrant further studies in order to obtain a correlation between enzyme subunits and their actions during transcription. In this study, a nucleoside triphosphate binding site was identified which is likely to be involved with catalysis. Only the use of more discriminating kinetics and the use of other nucleoside triphosphate labels can reveal whether peptide E carries domain(s) for the initiation and/or elongation reactions.

ACKNOWLEDGMENTS

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Registry No. 8-N_3ATP, 53696-59-6; 8-N_3GTP, 65114-35-4; RNA polymerase, 9014-24-8; UTP, 63-39-8; ATP, 56-65-5; dehydroxymethylamanitylazobenzoyl-*N*-glycylglycine, 99343-41-6.

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CORRECTIONS

Effect of Membrane Association on the Stability of Complexes between Ionophore A23187 and Monovalent Cations, by Richard W. Taylor, Clifford J. Chapman, and Douglas R. Pfeiffer*, Volume 24, Number 18, August 27, 1985, pages 4852–4859.

Page 4853. Equation 7 is incorrect and should read as follows:

$$[MA_b]/[A^-_b] = \left(\frac{\Delta F_{obsd}}{\Delta F_{max}}\right) \Big/ \left(1 - \frac{\Delta F_{obsd}}{\Delta F_{max}}\right) \quad (7)$$

Contribution of Monosaccharide Residues in Heparin Binding to Antithrombin III, by Donald H. Atha, Jean-Claude Lormeau, Maurice Petitou, Robert D. Rosenberg,* and Jean Choay, Volume 24, Number 23, November 5, 1985, pages 6723–6729.

Page 6725. In Table II, numerical values in the second row should read as follows: T (°C) = 6, ΔF_{max} (%) = 35, K_{diss} (M) = 1.0×10^{-8}, and $\Delta G°$ (kcal/mol) = 10.2. Due to a production error, these values were shifted one column to the right.