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PROBES OF EUKARYOTIC DNA-DEPENDENT RNA POLYMERASE II—I

BINDING OF 9-β-D-ARABINOFURANOSYL-6-MERCAPTOPURINE TO THE ELONGATION SUBSITE*

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Abstract—9-β-D-Arabinofuranosyl-6-mercaptopurine (ara-6-MP) was used to affinity-label wheat germ DNA-dependent RNA polymerase II (or B) (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6). This nucleoside analogue was found to be a competitive inhibitor with respect to [^{3}H]UMP incorporation. Natural substrates protected the enzyme from inactivation by ara-6-MP when the enzyme was preincubated with excess concentrations of substrates, suggesting that the inhibitor binds at the elongation subsite. The inhibitor bound the catalytic center of the enzyme with a stoichiometry of 0.6:1. The sulfhydryl reagent, dithiothreitol, reversed the inhibition by ara-6-MP, suggesting that the 6-thiol group of the inhibitor was interacting closely with an essential cysteine residue in the catalytic center of the enzyme. Chromatographic analysis of the pronase-digestion products of the RNA polymerase II–ara-6-MP complex also showed that ara-6-MP had bound a cysteine residue. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the denatured [6-^{35}S]ara-6-MP-labeled RNA polymerase II revealed that over 80% of the radioactivity was associated with the IIb subunit of the enzyme.

DNA-dependent RNA polymerase II (or B) catalyzes the transcription of exon plus intron genetic regions of DNA into promessenger RNA in eukaryotes. Very little is yet known about the catalytic center of this multisubunit eukaryotic enzyme but there is information which indicates that the prokaryotic and eukaryotic enzymes are mechanistically similar [1–3]. Based on the model of the catalytic center of the *Escherichia coli* enzyme proposed by us [4, 5], Krakow and Fronk [6], and Wu and Wu [7], a similar model for RNA polymerase II (or B) can be proposed. The catalytic center would be divided into three subsites: an initiation subsite, an elongation subsite, and a DNA template subsite. The initiation subsite would bind the initiating purine ribonucleoside 5′-triphosphates and later the 3′-end of a growing RNA chain. The elongation subsite would bind incoming ribonucleoside-5′-triphosphates for phosphodiester bond formation and the template subsite would bind DNA running in the 3′→5′ direction. This working model would allow for the design and synthesis of specific and potent inhibitors of the various subsites provided there were information on the microenvironment about each subsite. There have been reports on the binding of drugs to the catalytic center of eukaryotic RNA polymerase II although not specifically in the context of the above model. For instance, the α-amatoxins are known to block RNA chain extensions in eukaryotes [8, 9] and a radiolabeled derivative, [^{3}H]amanin, was used to affinity-label this site on calf thymus RNA polymerase II [10]. This implies that [^{3}H]amanin (and the α-amatoxins) is bound in or about the elongation subsite of the catalytic center. In addition, pyridoxal phosphate is reported to bind to an essential lysine in the catalytic center of rat liver RNA polymerase II although no subunit or subsite was specified [1].

We report here the use of an antitumor agent [11–13], 9-β-D-arabinofuranosyl-6-mercaptopurine (ara-6-MP)§, to probe the catalytic center of wheat germ RNA polymerase II. Ara-6-MP was found to bind close to an essential cysteine located in the elongation subsite found on the IIb subunit of the enzyme.

EXPERIMENTAL

Materials. [5,6-^{3}H]UTP (sp. act. 36 Ci/mmole) was purchased from ICN Pharmaceuticals Inc., Irvine, CA. [^{35}S]Sulfur (crystalline, rhombic, sp. act. 750 mCi/g) and L-[^{14}C]cysteine (sp. act. 30.2 mCi/mmole) were obtained from the Amersham Corp., Arlington Heights, IL. Nucleosides, nucleotides, calf thymus DNA, wheat germ (type I) and bovine serum albumin (crystallized and lyophilized) were purchased from the Sigma Chemical Co., St. Louis, MO. Tris base, $(NH_4)_2SO_4$ (enzyme grade) and urea (ultrapure) were obtained from Schwarz/Mann,

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§ Abbreviations: ara-6-MP, 9-β-D-arabinofuranosyl-6-mercaptopurine; DTT, dithiothreitol; TCA, trichloroacetic acid; and SDS, sodium dodecylsulfate.

Orangeburg, NY. Dithiothreitol, pronase and miracloth were purchased from the Calbiochem-Behring Corp., La Jolla, CA. Electrophoresis reagents (acrylamide, methylenebisacrylamide, *N,N'*-diallyltartardiamide, tetraethylmethylenediamine, ammonium persulfate, sodium dodecylsulfate, tracking dyes, bromophenol blue, Coomassie brilliant blue R-250, and mixed bed resin (AG 501-X8) were purchased from Bio-Rad Laboratories, Richmond, CA. The molecular weight markers for gel electrophoresis [thyroglobulin, ferritin, catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6), lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) and albumin] were obtained from Pharmacia Fine Chemicals Inc., Piscataway, NJ. All other chemicals of reagent grade were purchased from the Sigma Chemical Co.

Enzyme purification and standard assay. Wheat germ DNA-dependent RNA polymerase II was isolated and purified to homogeneity by the method of Jendrisak and Burgess [14]. The standard RNA polymerase II assay contained in a final volume of 0.25 ml: 2.5 μmoles Tris–HCl (pH 7.9); 5 μmoles $MgCl_2$; 0.25 μmole $MnCl_2$; 12.5 μmoles $(NH_4)_2SO_4$; 100 nmoles each of ATP, GTP and CTP; 1.0 μCi [5,6-^{3}H]UTP (sp. act. 1.0 μCi/nmole); 50 μg of heat-denatured calf thymus DNA; 125 μg of bovine serum albumin and various amounts of enzyme. The assay mixtures were incubated for 15 min at 25° and the acid-insoluble materials were precipitated by adding 2 ml of 5% (w/v) ice-cold TCA containing 25 mM sodium pyrophosphate. After 5 min in an ice-water bath, the precipitates were collected on Whatman glass fiber filters (GF/C, 2.4 cm) and washed five times under suction with 5 ml each time of 2% ice-cold TCA containing 10 mM sodium pyrophosphate and followed finally by 2 ml of 95% ethanol. The filters were then dried at room temperature, and radioactivities were measured in a Beckman liquid scintillation counter (LS9000) after the addition of 10 ml of toluene base scintillation solution to each vial.

For inhibition studies with ara-6-MP, the enzyme was preincubated for 10 min at 25° with various concentrations of inhibitor before the addition of substrates and DNA in a final volume of 0.25 ml. The reaction mixtures were then assayed as described above.

Synthesis of [6-^{35}S]ara-6-MP. Radiolabeling of ara-6-MP with rhombic sulfur-35 was carried out by the sulfur-exchange reaction [15, 16]. Ara-6-MP (150 mg) and 32 mg of sulfur-35 (25 mCi) in 25 ml of anhydrous pyridine were heated under reflux for 4 hr. Then 25 ml of H_2O was added, and the reaction mixture was taken to dryness *in vacuo*. The dried mixture was extracted with 10 ml of hot water and filtered while hot under vacuum through a sintered-glass funnel with a fine filter. The filtrate was again taken to dryness under vacuum and then extracted with 10 ml of hot water and filtered again through a fine sintered-glass funnel. This procedure of drying, extracting, and filtering was carried out a third time. Finally, the [^{35}S]ara-6-MP was recrystallized from hot water, collected, and dried *in vacuo* to yield 100 mg of radiopure [6-^{35}]ara-6-MP as determined by silica gel thin-layer chromatography [17]. The initial specific activity was 2.9×10^7 dpm/μmole (13.2 mCi/mmole).

Labeling of RNA polymerase II with [6-^{35}S]ara-6-MP. To determine the molar binding ratio, 300 μg (0.55 nmole) of enzyme was incubated with various concentrations of [6-^{35}S]ara-6-MP (from 0.1 to 5.0 mM) for 10 min at 25° in a final volume of 0.25 ml containing 0.1 M $KHCO_3$ buffer (pH 7.9), 8 μM $MgCl_2$ and 0.2 M KCl. The reaction mixtures were precipitated by adding 2 ml of 10% ice-cold TCA containing 25 mM sodium pyrophosphate. After 5 min in an ice-water bath, the labeled mixtures were collected on Millipore filters (RAWP, 25 mm), each washed five times with 5 ml each time of 2% ice-cold TCA containing 10 mM sodium pyrophosphate and then dried at room temperature. The radioactivity was measured in a liquid scintillation counter after adding 10 ml of toluene-based scintillant to each vial. Experiments for the determination of substrate protection against ara-6-MP inhibition were carried out by preincubating 22 pmoles of RNA polymerase II for 15 min at 25° with 400 nmoles each of ATP, GTP and CTP containing 2.5 μmoles Tris–HCl (pH 7.9), 0.25 μmole $MnCl_2$, 5 μmoles $MgCl_2$, and 12.5 μmoles $(NH_4)_2SO_4$ before adding ara-6-MP to the reaction mixture (in a final volume of 0.25 ml) as outlined above.

For irreversible binding (covalent bond formation) of [6-^{35}S]ara-6-MP to RNA polymerase II, the procedure of Miller *et al.* [17] was used which employs H_2O_2 to promote the formation of the disulfide bond. To 91 pmoles of RNA polymerase II in 25 μmoles Tris–HCl buffer (pH 7.9) containing 0.1 mM H_2O_2 was added [6-^{35}S]ara-6-MP (1 mM). Appropriate controls with and without H_2O_2 and [6-^{35}S]ara-6-MP were carried along in the experiments. The reaction mixtures were allowed to preincubate for specified times at 25° before the addition of substrates and DNA and then were assayed.

Protein concentrations were measured by the method of Lowry *et al.* [18] using crystalline bovine serum albumin as a standard. In addition, the protein concentration of pure wheat germ RNA polymerase II was determined by using a specific absorbance at 280 nm of 0.65 mg protein per ml of solution [19].

Polyacrylamide gel electrophoresis. Gel electrophoresis under nondenaturing conditions was run according to the procedure of Davis [20]. Sodium dodecylsulfate–gel electrophoresis was performed according to the method of Laemmli [21] with the slight modification of replacing methylenebisacrylamide with *N,N'*-diallyltartardiamide (for the hydrolysis of the gel) and reducing the concentration of β-mercaptoethanol by one-tenth. Gels were stained overnight in 0.05% Coomassie brilliant blue R-250 in ethanol–glacial acetic acid–water (5:1:5, v/v), and destained with magnetic stirring in 7% acetic acid containing approximately 10% (w/v) Bio-Rad mixed bed resin for 12 hr at room temperature until the gels cleared. The gels were scanned at 550 nm in a Beckman 25 spectrophotometer equipped with a gel scanning apparatus. For measuring the radiolabeled gels, the gels were cut into 2 mm slices and digested with 0.4 ml of 30% H_2O_2 in tightly capped scintillation vials at 55° for 5 hr. The vials were cooled to room temperature, 15 ml of ACS

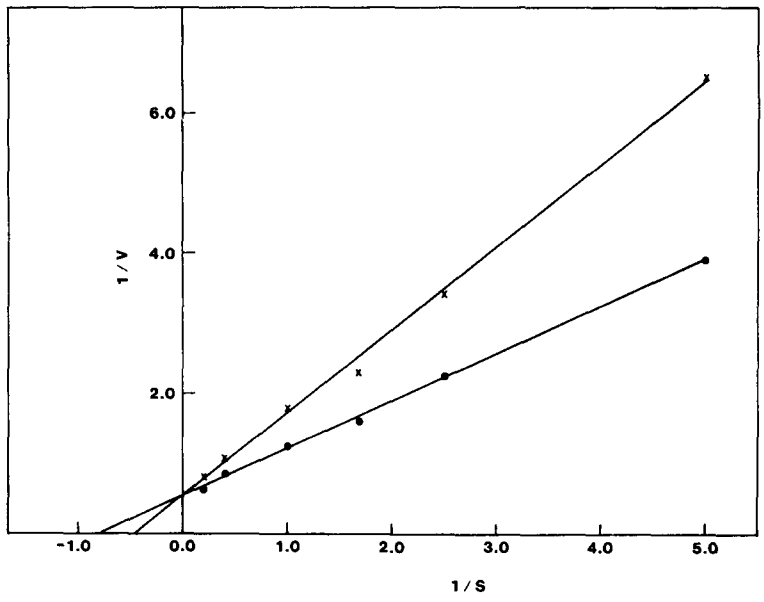

Fig. 1. Lineweaver–Burk plot of ara-6-MP inhibition. RNA polymerase II, 22 pmoles, in a final volume of 0.25 ml contained 2.5 μmoles Tris buffer (pH 7.9); 5 μmoles $MgCl_2$; 0.25 μmole $MnCl_2$; 12.5 μmoles $(NH_4)_2SO_4$; 100 nmoles each of ATP, GTP, and CTP; various concentrations of [5,6-^{3}H]UTP (1 μCi); 50 μg of heat-denatured calf thymus DNA; and 125 μg of bovine serum albumin; ara-6-MP (1 mM) was preincubated with the enzyme for 10 min at 25° before the addition of substrates and DNA. Final incubations were carried out for 10 min at 25° before assayed. The rate for all was measured as dpm incorporated into an acid-insoluble product in 15 min. *S* represents [UTP] × 10^{-3} M. Key: (●——●) controls; and (×——×) ara-6-MP (1 mM).

(aqueous counting scintillant, Amersham Corp.) was added and vortexed vigorously, and the samples were counted for radioactivity.

RESULTS

Inhibition of RNA polymerase II by ara-6-MP. Ara-6-MP inhibited DNA-dependent RNA synthesis catalyzed by wheat germ RNA polymerase II. A Lineweaver–Burk plot of ara-6-MP inhibition is depicted in Fig. 1 which shows that ara-6-MP is a competitive inhibitor of [^{3}H]UMP incorporation into RNA. The K_m (for [^{3}H]UTP) was determined to be 1.4 mM, while the K_i value for ara-6-MP was calculated to be 5.4 mM. Thus, the affinity of the enzyme for ara-6-MP is approximately one-fourth of that of UTP, and one would expect to see little if any inhibition by ara-6-MP of RNA synthesis *in vivo*. In this connection, at therapeutic dosage, ara-6-MP had no apparent effect on RNA synthesis in tumor cells [11]. At any rate, the results of the kinetic study carried out here indicated that ara-6-MP competed with UPT for binding to the catalytic center and implies that ara-6-MP was binding in or about the elongation subsite. The other three substrates for RNA synthesis (ATP, GTP, and CTP) provided protection from ara-6-MP binding (Table 1) as one might expect for competitive inhibition. When ara-6-MP was used at 2 mM concentration in these experiments, [^{3}H]UMP incorporation into RNA was inhibited 50%. However, if either one of the other three substrates was preincubated with the enzyme at a 0.8 mM concentration (0.4:1 ratio of substrate to inhibitor) before the addition of ara-6-MP at 2 mM, inhibitions were reversed about 30%. Preincubation of the enzyme with template afforded 10% protection.

Table 1. Substrate protection against the inhibition of wheat germ RNA polymerase II activity by ara-6-MP*

Reaction conditions	% of control activity	% protection
Control	100	
Ara-6-MP, 2 mM	50	
ATP + ara-6-MP	67	34
GTP + ara-6-MP	65	30
CTP + ara-6-MP	66	32
DNA + ara-6-MP	55	10

* RNA polymerase II (22 pmoles) was incubated for 10 min with nucleotides (0.8 mM) and DNA (0.4 mg/ml), and then for 10 min with ara-6-MP (2.0 mM) at 25° in 2.5 μmoles Tris–HCl (pH 7.9) containing 5 μmoles $MgCl_2$, 0.25 μmole $MnCl_2$ and 12.5 μmoles $(NH_4)_2SO_4$. After incubation, the reaction mixtures were assayed for enzyme activity as described in the Experimental section. Control activity was the incorporation of 2.4 × 10^3 dpm of [^{3}H]UMP into RNA in 20 min.

Effects of DTT and H_2O_2 on the ara-6-MP inhibition of RNA polymerase II. The effect of DTT on ara-6-MP inhibition of RNA polymerase II is shown in Table 2. When the enzyme was preincubated with ara-6-MP for 10 min and then 10^{-3} M DTT was added along with the substrates, the enzyme inhibition was reversed by about 30%. In contrast, when the

Table 2. Effect of DTT on the inhibition of RNA polymerase II by ara-6-MP*

Ara-6-MP (M)	Inhibition (%)		
	(a) 0 mM DTT	(b) 1 mM DTT	(c) 1 mM DTT preincubated
0	5.24	0	0
1×10^{-3}	67.42	39.10	6.71
5×10^{-3}	89.23	62.67	6.97

* RNA polymerase II (22 pmoles) was preincubated with various concentrations of ara-6-MP (a) without DTT, (b) with DTT for 10 min at 25°, or (c) preincubated with DTT for 5 min and then with ara-6-MP for 10 min at 25°. The resulting mixtures were assayed by the standard system as described in the Experimental section.

enzyme was preincubated with DTT before the addition of ara-6-MP, no inhibition of RNA polymerase II was observed. These results suggested the possibility that the 6-sulfhydryl group of ara-6-MP was close to a cysteine residue in or near the catalytic center of the enzyme and that possibly a disulfide bond between the enzyme and the inhibitor could be formed chemically. Evidence of disulfide-bond formation was observed by preincubating the enzyme with ara-6-MP and H_2O_2 and then assaying enzyme activities as a function of incubation time (Fig. 2). The inhibition of RNA polymerase II by ara-6-MP with H_2O_2 was progressive with incubation time, suggesting that disulfide-bond formation was promoted by H_2O_2 and that the enzyme was irreversibly inhibited.

Binding of ara-6-MP to the enzyme and identification of the bound subunit. From a Hill plot, it is possible to determine whether one, two or several inhibitor molecules are involved in binding to the catalytic center of an enzyme [22, 23]. This is depicted in Fig. 3 where the slope of ara-6-MP inhibition of [^{3}H]UMP incorporations was constant at 0.6 (or essentially one) over a broad range of ara-6-MP concentrations ranging from 0.1 to 5 mM. This indicated that one molecule of ara-6-MP was required to bind and inhibit one catalytic center of the enzyme. However, unessential (or nonsensitive) sites on the enzyme could have been bound by ara-6-MP at high concentrations and it was important to determine the one-to-one binding stoichiometry in preparation for the determination of the subunit

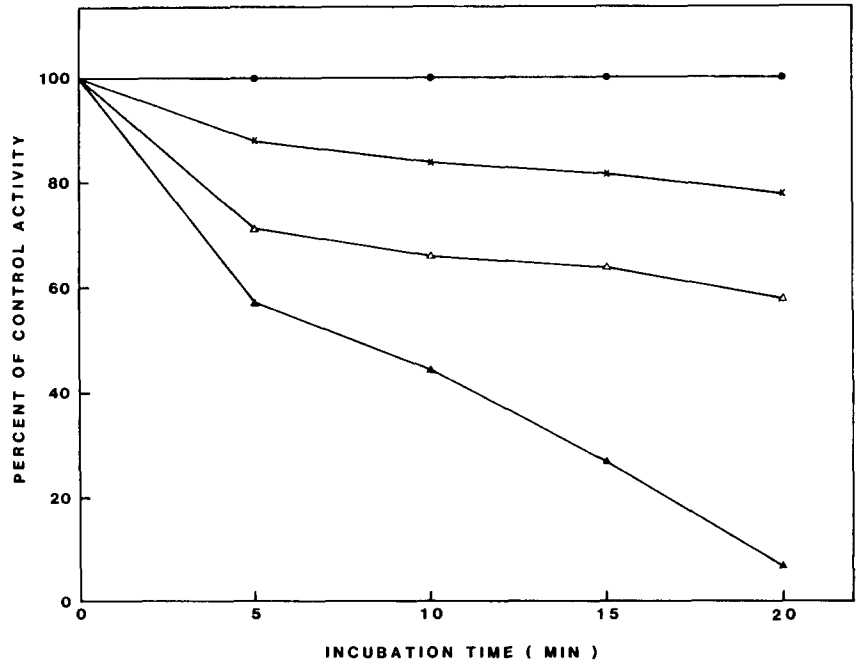

Fig. 2. Effects of H_2O_2 on ara-6-MP inhibition of RNA polymerase II. H_2O_2 (0.1 mM), ara-6-MP (1 mM), or the combination were preincubated with 91 pmoles of RNA polymerase II in a final volume of 1.0 ml containing 2.5 μmoles Tris buffer (pH 7.9) before the addition of substrates and DNA. At specified times, 0.1-ml aliquots were removed and assayed by the standard system as described in the Experimental section. Key: (●——●) control; (×——×) preincubated with 0.1 mM H_2O_2; (△——△) preincubated with 1 mM ara-6-MP; and (▲——▲) preincubated with 1 mM ara-6-MP and 0.1 mM H_2O_2.

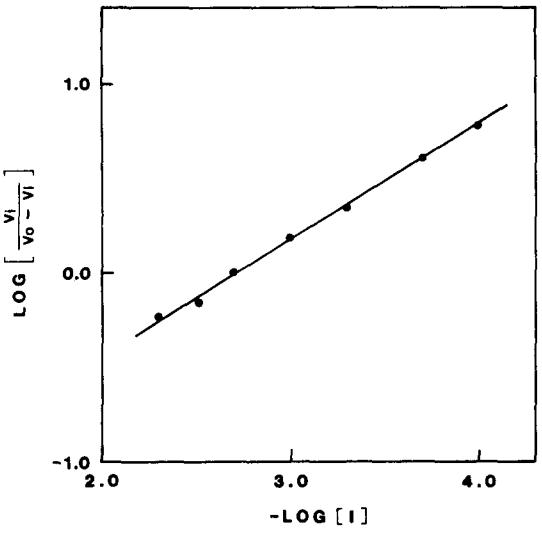

Fig. 3. Hill plot of the inhibition of wheat germ RNA polymerase II by ara-6-MP. The enzyme (24 pmoles) was preincubated with various concentrations of ara-6-MP ranging from 0.1 to 5.0 mM for 10 min at 25°. As described in the experimental section, all substrates and template in a final volume of 0.25 ml contained 2.5 μmole Tris–HCl buffer (pH 7.9); 5 μmoles $MgCl_2$; 0.25 μmole $MnCl_2$; and 12.5 μmoles $(NH_4)_2SO_4$; 125 μg of bovine serum albumin was added and then the samples were incubated for 15 min at 25°. The reaction was stopped and the RNA was precipitated, filtered, and counted as described in the Experimental section. Vo and Vi represent the uninhibited and inhibited reaction rates respectively. [I] indicates various inhibitor concentrations. The slope was calculated using a least squares computer program.

bound by ara-6-MP. The binding stoichiometry was determined by the filter-binding assay of Kimball [16] which is a biochemical application of the Freundlich adsorption isotherm [24]. The results are shown in Table 3. A log plot of these binding data gave a [6-^{35}S]ara-6-MP concentration of 0.28 mM that would bind 0.55 nmole of enzyme with a one-to-one stoichiometry. This [6-^{35}S]ara-6-MP-to-enzyme concentration ratio was used in the affinity-labeling of the enzyme and in the determination of the modified amino acid in the catalytic center. SDS–polyacrylamide gel electrophoresis of the RNA polymerase II–[6-^{35}S]ara-6-MP covalent complex promoted by H_2O_2 showed that the IIb subunit of the enzyme was bound by ara-6-MP (Fig. 4). Furthermore, pronase digestion of the labeled complex and subsequent paper and thin-layer chromatography (data not shown) of the digest [17] revealed that a cysteine had been modified by ara-6-MP through disulfide-bond formation.

DISCUSSION

The catalytic center of DNA-dependent RNA polymerase II from wheat germ as a model eukaryotic system was probed here with the antitumor agent ara-6-MP [11–13], in order to obtain information about the microenvironment surrounding the binding site. Previously, ara-6-MP had been used to affinity-label the elongation subsite of the *E. coli* enzyme [17], and our results appear to bear out the similarities in function of the prokaryotic and eukaryotic systems [1–3]. Since ara-6-MP was found to be a competitive inhibitor with respect to [^{3}H]UTP and in combination with other kinetic data, the binding of ara-6-MP is tentatively placed in or very near the elongation subsite of the catalytic center of the wheat germ enzyme. The reversal of inhibition by DTT and the promotion of a disulfide bond with H_2O_2 between the 6-thiol group of ara-6-MP and an essential cysteine on the enzyme indicate that the sixth position of the base moiety of ara-6-MP lies close to a cysteine composing part of the elongation subsite. This finding is not unique since similar observations have been made for guanosine-5′-monophosphate reductase [25] and adenosine triphosphatase of myosine [26] using mercaptopurine nucleoside phosphate compounds where disulfide-bond formation was found to occur. The K_m (UTP) for the enzyme and the K_i for ara-6-MP are of the

Table 3. Determination of the concentration at which [^{35}S]ara-6-MP binds in a 1:1 molar ration with RNA polymerase II*

RNA polymerase (nmole/reaction)	[^{35}S]Ara-6-MP		Molar binding ratio (ara-6-MP/enzyme)
	Concn (M)	nmoles bound	
0.55	1×10^{-5}	0.0178	0.0323
0.55	5×10^{-5}	0.0872	0.1585
0.55	1×10^{-4}	0.2140	0.3890
0.55	2×10^{-4}	0.3805	0.6918
0.55	5×10^{-4}	1.0242	1.8621

* Wheat germ RNA polymerase II (0.55 nmole) was incubated with various concentrations of [^{35}S]ara-6-MP in a 0.1 M $KHCO_3$ buffer (pH 7.9) containing 8 μM $MgCl_2$ and 0.2 M KCl for 10 min at 25°. The reaction mixtures were precipitated by adding 2 ml of 10% ice-cold TCA containing 25 mM sodium pyrophosphate. After 5 min at 0°, the labeled mixtures were collected on Millipore membrane filters (RAWP, 25 mm), each washed five times with 5 ml each time of 2% ice-cold TCA containing 10 mM sodium pyrophosphate and then dried at room temperature. The radioactivity was measured in a liquid scintillation counter after adding 10 ml of toluene-based scintillant to each vial.

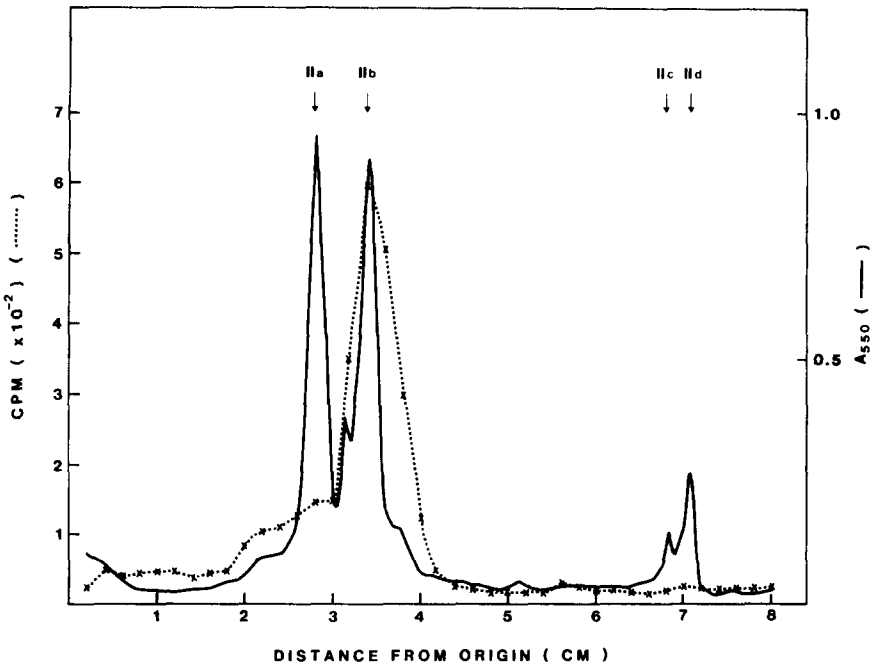

Fig. 4. SDS–gel electrophoresis after covalent binding of [^{35}S]ara-6-MP to wheat germ RNA polymerase II. The enzyme (0.55 nmole) was incubated with 0.28 mM [^{35}S]ara-6-MP in 0.1 M $KHCO_3$ buffer (pH 7.9) containing 8 μM $MgCl_2$ and 0.2 M KCl in a final volume of 0.5 ml for 10 min at 25°. H_2O_2 (1 mM) was added, and the sample was incubated for another 15 min at 25°. This mixture was denatured and electrophoresed in 5% polyacrylamide gel as described in the Experimental section. The solid line indicates the absorbance pattern at 550 nm after staining with Coomassie brilliant blue R-250; the broken line shows the radioactivity in a parallel gel. Key: subunit IIa, mol. wt 220,000; subunit IIb, mol. wt 140,000; subunit IIc, mol. wt 42,000; and subunit IId, mol. wt 40,000. Initial electrophoresis in 12.5% gels had shown that subunits below IId were unlabeled.

same order of magnitude; therefore, ara-6-MP could not be classified as a very potent inhibitor of RNA polymerase II. The 5′-triphosphate derivative would probably have a lower K_i since there is likely to be a 5′-triphosphate recognition site in the elongation subsite which would strengthen the force of binding. However, it is well known that phosphate derivatives of nucleosides do not go through cell membranes well and the long-range goal here is the development of extremely potent nucleoside inhibitors of RNA polymerase II as possible chemotherapeutic agents. The juxtaposition of the sixth position of the purine moiety of ara-6-MP to an essential cysteine in the catalytic center offers possibilities for the preparation of suicidal inhibitors of the enzyme—a chloroacetyl derivative of ara-6-MP or another purine nucleoside, for instance.

Eukaryotic DNA-dependent RNA polymerase II is a very complex enzyme composed of many subunits the number of which varies with the species, ranging from nine for the mouse plasmacytoma enzyme [27] to eleven for the wheat germ enzyme [28]. Previously, Brodner and Wieland [10] covalently bound an amatoxin derivative to the elongation subsite of calf thymus RNA polymerase II in molecular studies of that enzyme. We have outlined here an investigation of that subsite on the wheat germ enzyme employing an anticancer agent. Such studies are needed if specific and potent inhibitors are to be forthcoming.

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