Alterations in DNA-Dependent RNA Polymerases I and II from Rat Liver by Thioacetamide: Preferential Increase in the Level of Chromatin-Associated Nucleolar RNA Polymerase IB†

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ABSTRACT: The effect of a single injection of thioacetamide (50 mg/kg), a hepatocarcinogen, on the levels of rat liver RNA polymerases I and II, was investigated. The enzymes in the "bound" (chromatin-associated) and "free" forms were extracted separately and purified extensively by several column chromatographic fractionations. The total units of the bound RNA polymerases IA and IB (corresponding to the enzyme derived from the same DNA equivalent nuclei in the control and thioacetamide-treated liver) increased three- to fourfold within 24 h of administration of the carcinogen. The stimulation of bound RNA polymerase IB was significantly higher than that of bound IA. At this time point, the level of bound RNA polymerase II increased by only 30–50%, and that of free RNA polymerase I or II was relatively unaffected. The difference between the two enzymes became evident only when the template concentration was nonlimiting. Thioacetamide did not significantly alter the size of the product synthesized by bound polymerase I. Finally, when the enzyme assay was performed using similar units of the enzyme from the control and carcinogen-treated livers (rather than quantities of enzyme corresponding to the same DNA equivalent nuclei from these tissues) under conditions favoring one round of initiation, no significant difference in the level of the bound RNA polymerase I was observed. These data suggest that the effect of thioacetamide on RNA polymerase I is manifested by an increase in the amount of enzyme protein, rather than by activation of the preexisting enzyme molecules. The preferential increase in the level of bound RNA polymerase I over that of free enzyme is consistent with the proposed role of chromatin-associated polymerases in active transcription and might be a mechanism responsible for the augmented ribosomal RNA precursor synthesis observed after thioacetamide administration.

All eukaryotic cells investigated to date have been found to contain three classes of RNA polymerases[1] (for reviews, see Jacob, 1973; Chambon, 1975; Jacob and Rose, 1977) that are involved in the synthesis of specific classes of RNA. Although a specific regulatory role has not been established for eukaryotic RNA polymerases, there are suggestions that mammalian RNA polymerase(s) may modulate transcription. Studies on the probable role of RNA polymerases in the control of gene transcription in chemically induced carcinogenesis are complicated by the early cytotoxic effects of many of these compounds. An alternate approach to this problem is to select a compound which has a very limited early cytotoxic effect, and yet produces tumors in target tissues after prolonged treatment. One such agent appears to be thioacetamide (Hunter et al., 1977) which, in the first week of treatment, can induce enlargment of nucleoli (Busch and Smetana, 1970) and enhance ribosomal RNA synthesis in liver (Steele and Busch, 1966), both of which are characteristics of many tumors. Moreover, it has been shown to produce liver tumors (Fitzburgh and Nelson, 1948; Anghileri et al., 1977) after prolonged treatment. In the course of our investigation concerning the transcriptional events in the early stages of carcinogenesis, the effect of thioacetamide on class I and II RNA polymerases from liver was investigated. Relative to normal liver RNA polymerases IA and IB, the levels of the corresponding enzymes in the thioacetamide-treated liver were several-fold higher within 24 h of a single administration of the carcinogen, and the effect was relatively specific with respect to the transcriptionally active or chromatin-associated (bound) population of RNA polymerase IB.

Materials and Methods

Animals and Treatment. Sprague-Dawley rats were obtained as weanlings from Charles Rivers Laboratories. They were maintained under controlled conditions to weights between 120 and 150 g. At all times food and water were available ad libitum. Thioacetamide (50 mg/kg) was injected intraperitoneally in a 0.15 M NaCl solution (25 mg/mL). Control animals received 0.15 M NaCl by the same route.

Buffers. The standard buffer consisted of 50 mM Tris-HCl (pH 7.9 at 20 °C), 25% glycerol (v/v), 5 mM $MgCl_2$, 0.1 mM Na_2EDTA, and 0.5 mM dithiothreitol (TGMED). TED buffer contained 50 mM Tris-HCl (pH 7.9 at 20 °C), 0.1 mM Na_2EDTA, 0.5 mM dithiothreitol. TED-25 and TED-50 buffers were TED buffer containing 25 and 50% glycerol (v/v), respectively. In all cases, dithiothreitol was added just prior to use.

Isolation and Fractionation of Nuclei. Rats were sacrificed by decapitation and their livers were immediately removed and suspended in cold 0.25 M sucrose. Nuclei were isolated by homogenization of minced livers in 13 volumes of 2.3 M sucrose containing 3.3 mM $CaCl_2$ (Chauveau et al., 1956; Jacob et al., 1967). Replacement of $CaCl_2$ with 15 mM $MgCl_2$ (Busch et al., 1967; Jacob et al., 1969) did not alter the activity of the enzyme in the solubilized extract, although Mg^{2+} was

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[1] Abbreviations used: RNA polymerase, nucleotidetriphosphate:RNA nucleotidyltransferase (EC 2.7.7.6); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

preferrable when RNA polymerase was assayed in isolated nuclei. The recovery of nuclei was highly reproducible when $CaCl_2$ was used in the homogenization medium. Nuclei were sedimented by centrifugation at 40 000*g* for 60 min. Free and bound RNA polymerases were extracted from isolated nuclei essentially as described by Yu (1975). The nuclear pellet was resuspended gently in 0.25 M sucrose (1 g/mL) using a Teflon pestle and centrifuged at 20 000*g* for 10 min. The supernatant (nuclear supernatant or sap) represented the free RNA polymerases and the pellet contained bound RNA polymerases.

Solubilization and Purification of Nuclear RNA Polymerases. Chromatin-associated (bound) RNA polymerases from the nuclear pellet were solubilized, precipitated with $(NH_4)_2SO_4$ (0.42 g/mL), and reextracted, essentially as described by Rose et al. (1975, 1976). The nuclear supernatant was diluted with an equal volume of 50 mM Tris-HCl buffer (pH 8.9) containing 40% glycerol (v/v), 1.0 mM $MgCl_2$, 0.1 mM Na_2EDTA, 2 mM dithiothreitol, 50 mM KCl, and 0.5 mM phenylmethanesulfonyl fluoride. The free RNA polymerases were then precipitated directly with $(NH_4)_2SO_4$ (0.42 g/mL). The precipitate was suspended in TGMED buffer and dialyzed against the same buffer. The denatured proteins, if any, were removed by high-speed centrifugation. No enzyme activity was detected in the residue left after extraction of the bound or free RNA polymerases. In all the experiments, the enzyme was subjected to column chromatography immediately after dialysis.

The enzyme extracts corresponding to the bound or free RNA polymerases derived from 20 g of liver were loaded onto 10 × 0.9 cm (DEAE-Sephadex A-25) columns that had been previously equilibrated with TGMED containing 10 mM $(NH_4)_2SO_4$. After washing the columns with 1.5 column volumes of the same buffer, RNA polymerases were eluted with a linear gradient (10–550 mM $(NH_4)_2SO_4$–TGMED). Fractions of 0.7 mL were collected and 40-μL aliquots were taken to determine RNA polymerase activity. Under these conditions, negligible activity was detected in the wash fractions and all the polymerase activity was eluted by the salt in the gradient (Rose et al., 1975). The fractions containing RNA polymerase activity were pooled, bovine serum albumin was added (1 mg/mL) and the mixture was then dialyzed overnight against TED-50. The enzyme was either purified further or stored at −90 °C. When larger amounts of tissue were used (70 g), an 11 × 1.5 cm DEAE-Sephadex A-25 column was used and elution was accomplished using a stepwise gradient consisting of 1.5 column bed volumes each of 170 mM $(NH_4)_2SO_4$ and 400 mM $(NH_4)_2SO_4$ buffer. Fractions, 2 mL in volume, were collected and 40-μL aliquots were assayed for RNA polymerase activity and processed as above.

Phosphocellulose Chromatography. After dialysis, RNA polymerase I from the DEAE-Sephadex columns was adjusted to 25% (v/v) glycerol using TED buffer and applied to 10 × 0.9 cm phosphocellulose columns that had been equilibrated with TED-25 buffer. After washing with 2 column volumes of TED-25, RNA polymerase I was eluted using a linear gradient (10–400 mM $(NH_4)_2SO_4$ in TED-25). One-milliliter fractions were collected and 40-μL samples were analyzed for activity. Fractions containing RNA polymerase activity were pooled, bovine serum albumin was added (1 mg/mL), and then the mixture was dialyzed against TED-50 buffer.

Rechromatography on DEAE-Sephadex A-25. Dialyzed enzymes pooled from the phosphocellulose column were loaded onto a 8 × 0.9 cm DEAE-Sephadex A-25 column equilibrated with TED-25. The column was washed with 1.5 column bed volumes of TED-25 containing 0.01 M $(NH_4)_2SO_4$ and the enzymes were eluted with a linear gradient (10–400 mM $(NH_4)_2SO_4$ in TED-25). Fractions of 0.6 mL were collected and 40-μL aliquots were assayed for RNA polymerase activity. The fractions corresponding to RNA polymerase activity were pooled, bovine serum albumin was added (1 mg/mL), and the mixture dialyzed against TED-50 buffer and stored at −90 °C for further analysis. The enzyme at this stage of purification was free of nucleases.

DNA-Dependent RNA Polymerase Assay. RNA polymerase activity was assayed as described previously (Rose et al., 1976). A typical assay contained 1.5 mM $MnCl_2$, 3.3 mM NaF, 8 mM KCl, 60 mM Tris-HCl (pH 8.0), 0.5 mM dithiothreitol, 0.64 mM CTP, GTP, and ATP, 40 μM [^{3}H]UTP, 20 000 cpm/nmol (New England Nuclear, 35 Ci/mmol), 15 μg of calf thymus DNA (Worthington), and 20–40 μL of enzyme in a total volume of 120 μL. When purified enzymes were used, $(NH_4)_2SO_4$ was added to a final concentration of 40 mM for polymerase I and 100 mM for polymerase II (Rose et al., 1976). After incubation at 30 °C for 10 min, reactions were terminated by chilling and adding 100 μg of unlabeled UTP to each tube. Aliquots (100–120 μL) were spotted onto Whatman DE81 filters and processed as described previously (Rose et al., 1976). One unit of enzyme activity corresponds to 1 nmol of UMP incorporated/10 min at 30 °C.

RNA Synthesis without Reinitiation. A single round of RNA chain initiation and elongation was achieved by a modification of the procedure described for blocking reinitiation by RNA polymerase II (Cedar, 1975). This procedure was based on the method originally devised for *E. coli* RNA polymerase (Hyman and Davidson, 1970). Initial experiments were performed to determine the concentration of $(NH_4)_2SO_4$ required to inhibit initiation by RNA polymerase I which was found to be 160 mM. Reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 1.5 mM $MnCl_2$, 8 mM KCl, 3.3 mM NaF, 0.5 mM dithiothreitol, 0.50 mM ATP and GTP, varying concentrations of calf thymus DNA and 10–30 μL of RNA polymerase I (purified through three chromatographic columns) were preincubated at 37 °C for 15 min in a total volume of 100 μL. After formation of the initiation complex, $(NH_4)_2SO_4$, CTP, and [^{3}H]UTP (60–80 cpm/pmol) were added to final concentrations of 160 mM, 0.50 mM, and 27 μM, respectively. The reaction mixture (120 μL total volume) was further incubated for 25 min at 37 °C and terminated by chilling and by adding 100 μg of unlabeled UTP. UMP incorporation was determined as described previously (Rose et al., 1976).

Estimation of Size of RNA Synthesized in Vitro. The size of RNA synthesized in vitro was determined by two procedures.

(a) Average Chain Length. RNA synthesized by RNA polymerase I during a single round of elongation was precipitated with trichloroacetic acid. The product was hydrolyzed for 18 h with 0.5 N KOH and subjected to thin-layer chromatography (Randerath and Randerath, 1967) on poly(ethylenimine)-cellulose as described by Rose and Jacob (1976). Using this method, uridine and UMP could be separated and average chain length estimated by the ratio, radioactivity in UMP + uridine/radioactivity in uridine.

(b) Sucrose Density Gradient Centrifugation. This was done essentially as described by us previously (Jänne et al., 1975). RNA was synthesized using the standard reaction mixture and the reaction was terminated by adding 100 μg of UTP. After adjustment to 25 mM NaOAc (pH 5.0), 25 mM EDTA, and 0.5% sodium *N*-laurylsarcosinate (Prescott et al., 1971), the samples from 10 reaction mixtures were incubated at 4 °C for 30 min, pooled, and centrifuged for 20 min at 27 000*g*. The

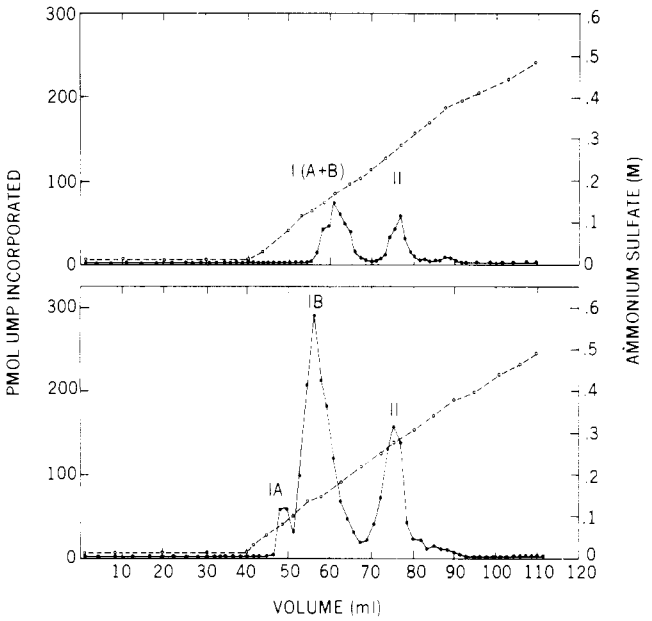

FIGURE 1: Effect of a single injection of thioacetamide (50 mg/kg) on the bound RNA polymerases I and II. The bound RNA polymerases were extracted from control (upper panel) and thioacetamide-treated (lower panel) rats (20 g liver each). The extraction of RNA polymerases, their fractionation on DEAE-Sephadex columns, and enzyme assay were performed as described in the text. Fractions corresponding to RNA polymerases I and II were individually pooled and assayed under optimal conditions as described in Materials and Methods.

supernatant was heated to 70 °C for 5 min (Ojala and Attardi, 1974) and 1-mL aliquots were layered over 2–20% sucrose density gradients containing 0.1 M NaCl, 10 mM NaOAc (pH 5.0), 1 mM EDTA, and 0.3% sodium *N*-laurylsarcosinate and centrifuged at 4 °C for 12 h at 38 000 rpm in a Beckman SW40 rotor. *E. coli* tRNA and rat liver ribosomal RNA (18S and 28S) were run as markers on parallel gradients. Gradients were fractionated and 0.35-mL fractions were collected. The fractions were spotted on Whatman DE81 filters and processed as described previously (Rose et al., 1976).

Estimation of Protein and DNA. Protein was estimated either by a modified Lowry's procedure (Bennett, 1967) or by the method of Schaffner and Weissman (1973). DNA was measured essentially as described by Burton (1956).

Results

Changes in the Levels of Bound and Free RNA Polymerases I and II in Liver following Thioacetamide Treatment. In the initial studies, RNA polymerases were extracted from whole nuclei isolated from control and thioacetamide-treated livers. Fractionation on a single DEAE-Sephadex column indicated an increase in RNA polymerase activity in carcinogen-treated animals. These studies showed that this increase was specific to the bound enzyme. Consequently, RNA polymerases were extracted from the nuclear residue and supernatant fractions, separately. Figure 1 (upper panel) shows the DEAE-Sephadex column chromatographic profile of bound RNA polymerases from normal liver. Two major peaks corresponding to RNA polymerases I and II and a minor peak corresponding to polymerase III were resolved. The three enzymes were characterized by their differential sensitivities to α-amanitin. Thus, polymerase I was insensitive to the toxin, whereas polymerases II and III were sensitive to low (0.5 μg/mL) and high (130 μg/mL) concentrations of the inhibitor, respectively. The lower panel in Figure 1 shows the effect of thioacetamide on chromatin-bound RNA polymerases. RNA polymerase IB was

TABLE I: Effect of Thioacetamide Pretreatment on RNA Polymerases I and II after a Single DEAE-Sephadex Chromatography.[a]

	Bound		Free	
	I	II	I	II
Control	2.53	9.58	1.0	10.1
Thioacetamide	7.8	13.4	1.5	13.5

[a] Enzymes were extracted from livers of control and thioacetamide-treated (20 g each) rats. Fractions containing the respective RNA polymerases obtained after a single DEAE-Sephadex column chromatography were pooled separately, dialyzed, and assayed with optimum salt concentrations as described in Materials and Methods. The values represent total units of enzyme (1 unit = 1 nmol of UMP incorporated/10 min at 30 °C) derived from 20 g each of control and thioacetamide-treated liver.

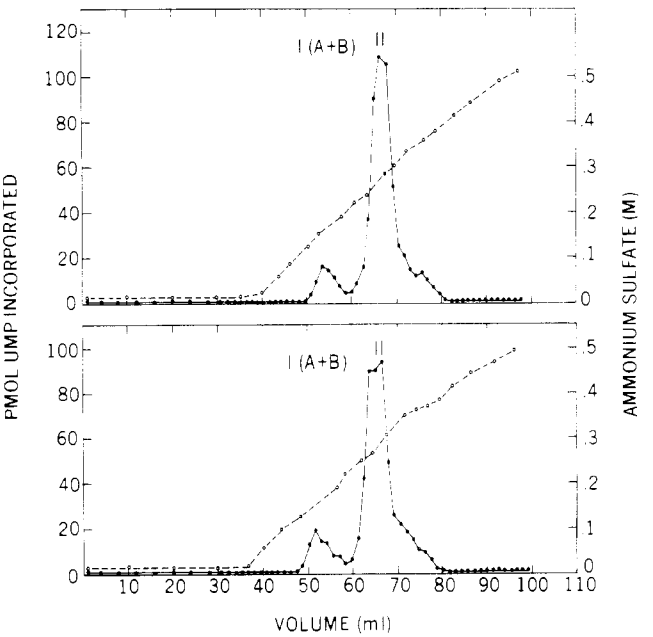

FIGURE 2: Effect of a single injection of thioacetamide (50 mg/kg) on the free RNA polymerases I and II. The free RNA polymerases were extracted from control (upper panel) and thioacetamide-treated (lower panel) rats (20 g liver each). The extraction of RNA polymerases, their fractionation on DEAE-Sephadex columns, and enzyme assay were performed as described in the text. Fractions corresponding to RNA polymerases I and II were individually pooled and assayed under optimal conditions as described in Materials and Methods.

significantly stimulated following treatment with the drug. As shown in Table I, the total units of bound polymerase I were 3-fold higher after thioacetamide treatment whereas only a 1.4-fold stimulation of RNA polymerase II activity was observed under optimal enzyme assay conditions.

The effect of thioacetamide on the free RNA polymerases present in the nuclear supernatant fraction was also investigated. Figure 2 shows the DEAE-Sephadex column chromatographic profiles of free enzymes from control and drug-treated animals. Contrary to the bound RNA polymerase, the levels of free RNA polymerase I and II were relatively unaltered in response to thioacetamide. As in the case of the bound enzymes, α-amanitin was used to identify the free RNA polymerases. Analysis of the pooled and dialyzed fractions under optimal conditions indicated (Table I) that thioacetamide increased the levels of free RNA polymerases I and II approximately 1.5-fold. Comparison of the effect of thioacetamide on bound and free enzymes showed that bound poly-

TABLE II: Persistent Stimulation of RNA Polymerase I by Thioacetamide through Enzyme Purification.[a]

Stages of purification	Enzyme activity: Control	Enzyme activity: Thioacetamide
$(NH_4)_2SO_4$-precipitated enzyme after dialysis[b]	139	308
1st DEAE-Sephadex	39	150
Phosphocellulose	13	45
2nd DEAE-Sephadex	2	8

[a] Animals were given a single dose of thioacetamide 24 h prior to sacrifice. Enzyme purification and assay were carried out as described in Materials and Methods. The enzyme activity corresponds to total units (1 unit = 1 nmol of UMP incorporated/10 min at 30 °C) derived from 70 g each of control and thioacetamide-treated liver. [b] RNA polymerase I activity corresponds to enzyme activity resistant to 130 μg/mL of α-amanitin. It should be noted that, at this stage of enzyme preparation, the stimulation of RNA polymerase activity by thioacetamide might be slightly masked by the presence of nucleolar RNase activity which is known to increase after the drug treatment (Busch and Smetana, 1970). The assay of individual RNA polymerase activities in total enzyme extract prior to chromatography should be considered as the best approximation to the absolute values.

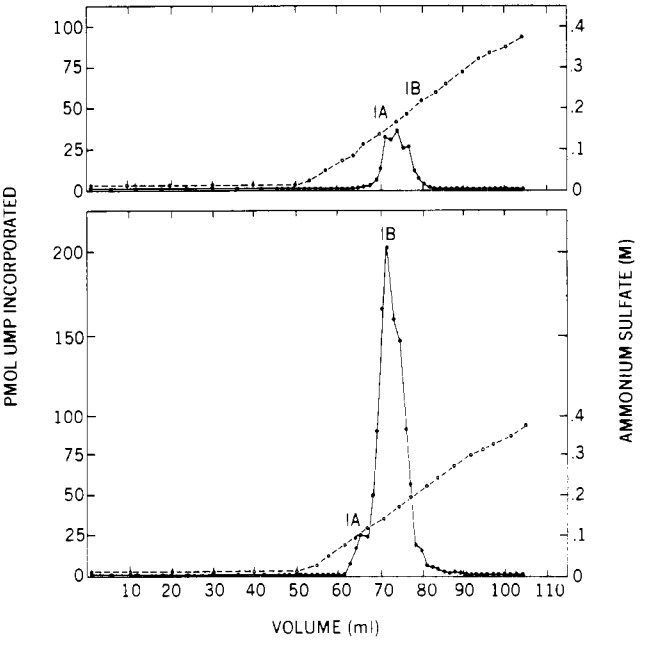

FIGURE 3: Final DEAE-Sephadex chromatography of the bound RNA polymerase I from control and thioacetamide-treated liver. The bound RNA polymerases were extracted from 70 g each of control (upper panel) and carcinogen-treated (lower panel) liver. RNA polymerase I (IA + IB) fractions were pooled separately, purified further through phosphocellulose column chromatography, and finally through a second DEAE-Sephadex chromatography as described in Materials and Methods.

merase I was preferentially increased. Since no decrease in free RNA polymerase I occurred, these data eliminated the possibility that the increase in bound polymerase was merely due to a redistribution of the two populations of enzyme. No attempts were made to investigate the effect of thioacetamide on RNA polymerase III activity.

In another set of experiments, enzymes were extracted from 25 g each of normal and drug-treated liver and from the same quantity of liver obtained by mixing equal proportions of normal and test tissues (12.5 g of control plus 12.5 g of treated). The total units of bound RNA polymerase I from these samples were 2.5, 7.7, and 4.7, respectively. Since the data obtained from the mixing experiments were very close to the theoretical value, i.e., 5.1 units, it is unlikely that the observed increase in bound polymerase I was due to the presence of inhibitory factors in the control enzyme or of stimulatory factors in the test enzyme preparations. To test further that the stimulatory effect of thioacetamide persisted through enzyme purification, bound RNA polymerase I was prepared using larger quantities of liver from control or thioacetamide-treated animals and purified through three successive chromatographic columns. Figure 3 shows the elution profile from the final (DEAE-Sephadex) column. It can be seen that the activity of RNA polymerase I, in particular enzyme IB, was substantially stimulated after thioacetamide treatment. Table II further shows the persistence of the stimulation of bound RNA polymerase I activity through three different chromatographic fractionations.

RNA Polymerase I Activity in the Absence of Reinitiation. Use of high salt has been successfully employed to prevent reinitiation by both *E. coli* RNA polymerase (Hyman and Davidson, 1970) and calf thymus RNA polymerase II (Cedar, 1975). The present method, adapted from Hyman and Davidson (1970), consisted of preincubation of the enzyme (in excess) and template with ATP and GTP in order to form the initiation complex. The addition of labeled UTP and high levels of $(NH_4)_2SO_4$ allowed RNA chain elongation but prevented reinitiation (see Materials and Methods). Preliminary experiments using varying concentrations of $(NH_4)_2SO_4$ during formation of the initiation complex indicated that 160 mM $(NH_4)_2SO_4$ could completely prevent initiation of RNA polymerase I from both control and drug-treated animals. In subsequent experiments, reinitiation was blocked with 160 mM $(NH_4)_2SO_4$.

TABLE III: Bound RNA Polymerase I Activity with Varying Amounts of DNA.[a]

DNA concn (μg/mL)	Control liver	Thioacetamide-treated liver
1	2.9	2.9
2	8.2	9.2
4	15.2	18.7
8	22.5	41.3

[a] Enzyme assay was performed under conditions inhibitory to reinitiation as described in the text. Equal volumes of bound RNA polymerase I, corresponding to the same DNA-equivalent nuclei from the control and thioacetamide-treated liver, were employed. Enzyme activity is expressed as pmol of UMP incorporated/25 min at 37 °C. The activities of both enzymes were linear for at least 10 min and reached a plateau at 20 min.

The bound RNA polymerase I from control and test animals (corresponding to the same DNA-equivalent nuclei), obtained after three column chromatographic fractionations, was assayed using DNA at concentrations ranging from 1 to 8 μg/mL (Table III). At 1 and 2 μg/mL of DNA, which were clearly limiting, the enzyme activity was identical in control and thioacetamide-treated samples. However, at higher DNA concentrations, the difference between control and test samples became evident. At 8 μg/mL of DNA, approximately twofold difference was noted between the two enzymes. These data suggest that thioacetamide-treated liver contains more enzyme molecules than the control liver and consequently requires more DNA for saturation. This was further tested by using the same units of enzyme activity (rather than the enzyme from the same DNA-equivalent nuclei) and varying amounts of DNA. As in the previous experiment, the initiation reaction

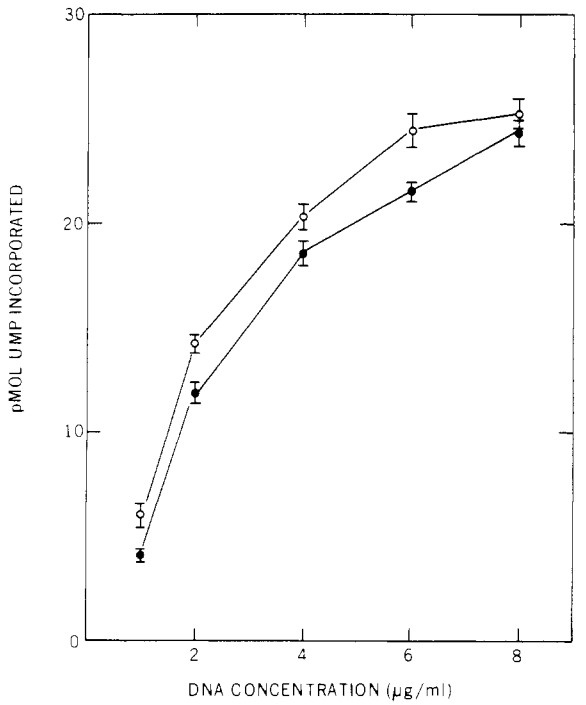

FIGURE 4: Effect of varying DNA concentration on the activity of similar units of bound RNA polymerase I from control and thioacetamide-treated liver, under conditions favorable for a single round of initiation and elongation. Aliquots of purified RNA polymerase I from control (●—●) and thioacetamide-treated liver (○—○) corresponding to 25 units of activity were assayed using excess DNA as template. Enzyme assay conditions have been described in Materials and Methods.

was performed by preincubating the enzyme and template without salt in the presence of purine nucleotides, followed by elongation without reinitiation in the presence of 160 mM $(NH_4)_2SO_4$. Since only a small difference was observed between control and thioacetamide-treated samples under these conditions (Figure 4), these experiments were indicative of increased amounts of enzyme protein in response to thioacetamide, rather than "activation" of preexisting enzymes by some chemical modifications such as phosphorylation.

Estimation of Size of Reaction Product. If the difference between the control and thioacetamide-treated enzymes was due to modifications in the enzyme molecules, it is possible that the enzyme derived from carcinogen-treated animals might facilitate a chain elongation reaction resulting in a longer product. To test this possibility, the size of the product synthesized in vitro by control and test enzymes was determined by measuring the average chain length after alkaline hydrolysis (see Materials and Methods). The average chain length determined by alkaline hydrolysis yielded 85 nucleotides for control liver enzyme and 86 nucleotides for the enzyme derived for thioacetamide-treated liver. The chain length was also independent of the units of enzyme used. Consequently, although the enzymes derived from the same DNA-equivalent nuclei from control and carcinogen-treated liver were used, the average chain length was similar. It should also be noted that time-course studies using similar units of enzyme for control and test enzyme, under conditions prohibiting reinitiation, did not show any difference between the two enzymes with respect to the rate of the elongation reaction. Analysis of the size of the reaction product by sucrose gradient centrifugation also indicated that the RNA synthesized by RNA polymerase I from control or thioacetamide treatment was similar. Figure 5 shows the gradient profiles for the products using the standard reaction conditions. RNA synthesized from both control and test enzymes sedimented at approximately 9 S.

Discussion

The present studies have demonstrated that thioacetamide, a hepatocarcinogen, can preferentially stimulate (three- to four-fold) the activity of partially purified bound nucleolar RNA polymerase IB. Recent studies using enzymes extracted from isolated nucleoli have substantiated these data (Leonard and Jacob, unpublished observation). Initial experiments showed that the enzyme activity was reduced by 20% in the first few hours, which is probably due to the early toxic effects of the compound. The 24-h time point was chosen because the most significant changes in RNA polymerases were observed at this time. Moreover, increases in the labeling of several RNA species in vivo have been observed within this time period (Ro-Choi et al., 1976). It should be emphasized that at several earlier time points examined, the augmentation of RNA polymerase I activity in the nuclei (identified by its insensitivity to high levels of α-amanitin) was less than that observed at the 24-h point but was always greater than the stimulation of RNA polymerase II (identified by its sensitivity to low levels of α-amanitin). This was also the case when the levels of RNA polymerases were measured after 9 days of repeated injections (50 mg kg^{-1} day^{-1}) with thioacetamide.

An interesting aspect of this investigation was the selective increase in the level of RNA polymerase IB relative to IA. Two forms of RNA polymerase I have been reported in a variety of eukaryotic cells (for a review, see Jacob, 1973; Chambon, 1975). The subunit composition of these forms appears to be different at least with respect to one subunit (Schwartz and Roeder, 1974; Roeder et al., 1975; Gissinger and Chambon, 1975; Matsui et al., 1976a). The possibility that these forms were artifacts produced during enzyme extraction and purification has been ruled out in a recent investigation (Matsui et al., 1976a; Kellas et al., 1977). These studies have also presented evidence which argued against the interconversion of these two forms during enzyme preparation. The selective increase in the level of RNA polymerase IB following thioacetamide treatment was also consistent with the finding that this enzyme was the transcriptionally active nucleolar enzyme in vivo (Matsui et al., 1976b).

The increased levels of RNA polymerase I in livers from the thioacetamide-treated rats do not appear to be due to a preferential recovery of the enzyme for the following reasons: (a) the stimulatory effect of thioacetamide was evident in the solubilized enzyme extract (activity resistant to high levels of α-amanitin) prior to column chromatography; (b) the total proteins in the thioacetamide-treated liver nuclei or in enzyme extract increased at most by 30–50% which might account for the increase in RNA polymerase II activity but is not sufficient to explain the three- to four-fold increase in enzyme I; (c) the three column chromatographic fractionations did not significantly change the percent stimulation of RNA polymerase I by thioacetamide which also rules out preferential inactivation of the control enzyme during purification; (d) the level of bound RNA polymerase I extracted after mixing equal amounts of tissue from the control and thioacetamide-treated livers was very close to the theoretical value; (e) finally, since the residue in the control and test samples, which remained after enzyme extraction, did not contain detectable enzyme activity, it seems unlikely that thioacetamide enhances the extractibility of bound enzyme I.

The mixing experiments also suggest the absence of any specific chromatin-associated factor(s) which may have contributed to the selective increase in the bound enzyme activity in vitro. These findings imply that thioacetamide exerts its

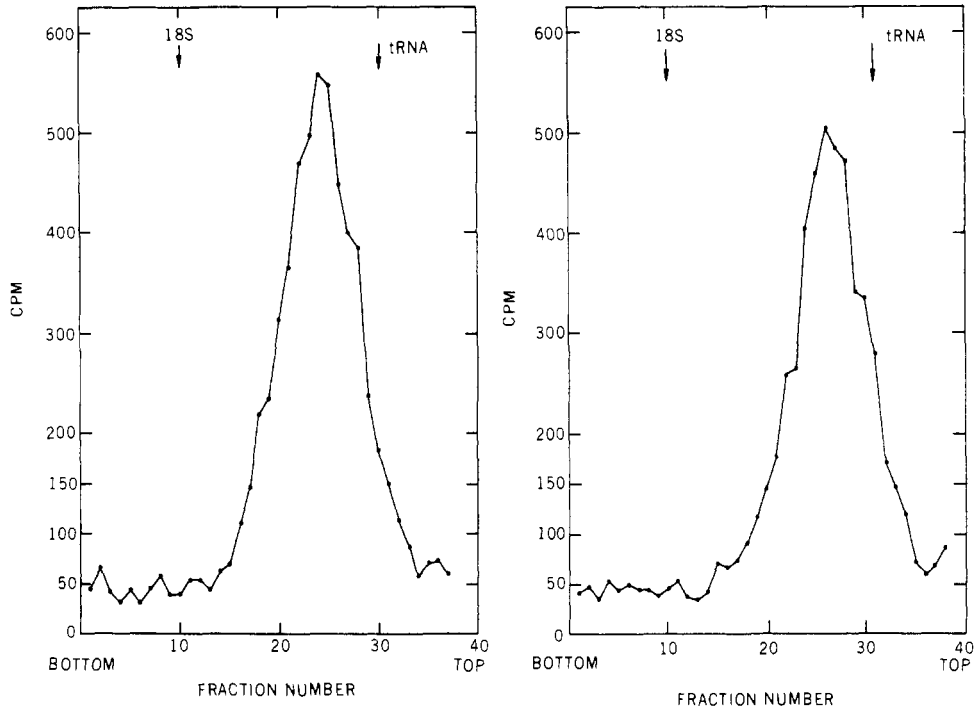

FIGURE 5: Sucrose density gradient sedimentation analysis of RNA synthesized in vitro. RNA was synthesized using calf thymus DNA (in excess) and purified bound RNA polymerase I from control (left panel) and thioacetamide-treated liver (right panel). The product analysis was performed as described in Materials and Methods.

effect after being metabolized in vivo. Indeed, Hunter et al. (1977) have recently demonstrated that the pathological effects of thioacetamide were secondary to its metabolism in vivo. The drug also had no direct effect on transcription when added in vitro (Leonard and Jacob, unpublished observations).

It may be argued that the higher activity of the bound RNA polymerase is due to a shift of the free enzyme population to the bound state. Since the bound RNA polymerase I has been postulated to be the transcriptionally active form of the enzyme (Matsui et al., 1976b), it is conceivable that a shift of free enzyme population to the bound state could occur when the cell is in need of larger quantities of RNA . However, no such redistribution of the two enzyme populations was observed after thioacetamide treatment, at least at the 24-h time point. In fact, the levels of free enzyme increased slightly after thioacetamide treatment.

The stimulation of RNA polymerase activity could occur by (1) increase in the extent of RNA chain elongation, (2) enhanced rate of reinitiation on the template, and (3) increase in the number of RNA polymerase molecules. The first two possibilities are conceivable if the enzyme had undergone chemical modifications (activation) such as phosphorylation (Hirsch and Martello, 1976). Since no detectable change in the product size has been observed after thioacetamide administration, it seems unlikely that the stimulatory activity is due to increased chain elongation. In addition these studies were performed under conditions inhibitory to reinitiation, and hence the higher activity observed at saturating quantities of template must not have resulted from an increased rate of reinitiation by the thioacetamide-treated enzyme. When the enzyme assay was performed using the same units (nmol of UMP incorporated) of the enzymes under conditions favorable for one round of initiation, no significant difference could be observed in the activities of the two enzymes (Figure 4), which further suggests that the two enzymes are qualitatively similar (Figure 4). Consequently, the absence of any difference in the activity of the enzymes from the control and carcinogen-treated livers at limiting DNA concentration coupled with the expression of higher activity by the latter enzyme at nonlimiting levels of DNA (Table III) indicate that thioacetamide induces an increase in the number of RNA polymerase molecules.

It is of interest to note that RNA polymerase IB activity has been shown to be stimulated in a Morris hepatoma (Rose et al., 1976), in liver following partial hepatectomy (Matsui et al., 1976b), and in hydrocortisone-treated liver (Sajdel and Jacob, 1971; Jacob et al., 1975). The early changes in the nucleolar RNA polymerase activity in the hydrocortisone-treated rats appeared to be due to an increase in the extent of chain elongation, as evidenced by the longer size of the product (Jacob et al., 1975b). A similar conclusion has also been reached by Barry and Gorsky (1971) when the effect of estradiol on RNA polymerase I activity was measured in isolated uterine nuclei. Thus, it appears that the early changes in RNA polymerase I activity in response to steroid hormones are different from those observed after thioacetamide treatment. However, further studies involving immunoprecipitation with antiserum against purified RNA polymerase I are required to prove unequivocally that the increased levels of this enzyme in the latter case are entirely due to an increase in the number of enzyme molecules which might have resulted from synthesis of new enzyme and/or from slower turnover rates of preexisting enzyme molecules.

It is noteworthy that the stimulation of RNA polymerase I activity after thioacetamide administration paralleled the increased labeling of ribosomal RNA precursor (Steele et al., 1965) and might be related to the increase (45%) in RNA polymerase activity in isolated nucleolar preparations (Villalobos et al., 1964). The augmented levels of RNA polymerase I might be the predominant mechanism for the increased ribosomal RNA synthesis which gets accumulated in the nucleolus after prolonged treatment with thioacetamide (Steele and Busch, 1966). The enlarged nucleolus induced by this agent may in turn be related to the accumulation of ribosomal RNA in this organelle. In this context, it may be pointed out

that previous studies in our laboratory have also shown that, in a rapidly growing hepatoma, the levels of RNA polymerase I, particularly IB, are higher than those in the host liver (Rose et al., 1976). It is conceivable that alterations in the bound RNA polymerase IB levels observed after thioacetamide administration may indeed be one of the important early biochemical events in the liver which might "trigger" cellular proliferation leading to neoplasia.

Acknowledgments

We wish to thank Dr. Y. C. Lin for helping with the standardization of the elongation reaction without reinitiation and Dr. K. M. Rose for useful discussion.

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