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SOLUBILIZATION AND CHARACTERIZATION OF RNA POLYMERASE FROM A HIGHER PLANT

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Abstract—RNA polymerase has been solubilized from sugar beet chromatin With calf thymus or sugar beet DNA as template, enzyme activity was linear with respect to protein concentration and required the presence of all four nucleoside triphosphates, added DNA and divalent metal ions The enzyme exhibited a sharp Mn^{2+} optimum of 1 25 mM and a Mg^{2+} optimum at 10 mM The Mn^{2+}/Mg^{2+} activity ratio (activity at optimum concentrations) was 2 0 with an optimum salt concentration of 50 mM Based on data including inhibition with α-amanitin (0 025 μg/ml), the majority of the total activity appeared to be RNA polymerase I Subsequent fractionation by DEAE-Sephadex column chromatography resulted in one peak of activity eluted with 0 18 M $(NH_4)_2SO_4$

INTRODUCTION

BOTH the type of RNA formed and the rate of RNA synthesis during development have been shown to vary with stages of differentiation [1,2] Such fluctuations in RNA synthesis may result from changes in chromatin[3] such as alterations in the DNA template available for transcription Based on evidence from several laboratories,[4,5] however, regulation of the synthesis and activity of RNA polymerase may also result in the observed changes in RNA synthesis For example, multiple RNA polymerases have been shown to exist in several eukaryotic organisms[6–9] and the presence of certain of these enzymes has been shown to vary during development [4] Therefore, an investigation of RNA synthesis in sugar beet tissue has included a study of chromatin-bound RNA polymerase activity

An earlier study[10] indicated that γ-rays inhibited chromatin-directed RNA synthesis possibly by inactivation of RNA polymerase and/or alteration of the DNA template. If the RNA polymerase from chromatin could be solubilized, it would then be possible to separate the effects of radiation on the enzyme from those altering the DNA template Since chromatin-bound RNA polymerase activity increases during washing (aeration)

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[2] WHITELEY, A H, MCCARTHY, B J and WHITELEY, H R (1966) *Proc Natl Acad Sci U S* **55**, 519
[3] BONNER, J, DAHMUS, M E, FAMBROUGH, D, HUANG, R C, MARUSHIGE, K and TUAN, D Y H (1968) *Science* **159**, 47
[4] ROEDER, R G and RUTTER, W J (1970) *Biochemistry* **9**, 2543
[5] TRAVERS, A (1971) *Nature New Biol* **229**, 69
[6] ROEDER, R G and RUTTER, W J (1970) *Proc Natl Acad Sci U S* **65**, 675
[7] HORGEN, P A and GRIFFIN, D H (1971) *Proc Natl Acad Sci U S* **66**, 338
[8] ROEDER, R G, REEDER, R H and BROWN, D D (1970) *Cold Spring Harbor Symp Quant Biol* **35**, 727
[9] POLYA, G M and JAGENDORF, A T (1971) *Arch Biochem Biophys* **146**, 635
[10] DUNHAM, V L, JARVIS, B C, CHERRY, J H and DUDA, C T (1971) *Plant Physiol* **47**, 771.

of sugar beet root tissue,[11] solubilization of the enzyme would also allow a detailed comparison of the enzyme from washed and unwashed tissue The purpose of this study was to solubilize RNA polymerase from chromatin and to determine the metal ion and salt requirements In addition, experiments were performed utilizing various inhibitors of specific RNA polymerases to determine the possible presence of multiple polymerases in the solubilized enzyme preparation

RESULTS

Solubilization of RNA Polymerase from Chromatin

To determine a procedure which would routinely solubilize the most activity from chromatin and still maintain an active RNA polymerase, three different procedures were compared (Table 1) As can be seen, all three procedures removed 80% of the total chromatin-bound RNA polymerase activity The specific activity of the solubilized enzyme, however, was the highest when the stirring procedure was employed In the sonication and homogenization experiments the solution frothed easily, and this probably resulted in enzyme denaturation The remainder of the experiments reported here utilized soluble RNA polymerase obtained by the stirring procedure

TABLE 1 SOLUBILIZATION OF RNA POLYMERASE FROM CHROMATIN

Procedure	% of chromatin activity remaining on pellet	Specific activity of soluble enzyme*
Sonication	21	5
VirTis homogenizer	23	17
Magnetic stirrer	20	25

* pmol of ^{3}H-UMP incorporated/min/mg protein

Two procedures for solubilization of enzyme from chromatin were compared to that described in Experimental (magnetic stirrer) Chromatin from 600 g of 20 hr washed tissue was either sonicated for a total time of 1 min (in 10 sec intervals to reduce frothing) in the presence of 0 3 M $(NH_4)_2SO_4$ with a Branson S125 (microtip attachment, setting 3), or homogenized in the presence of 0 3 M $(NH_4)_2SO_4$ for 30 min in a 5-ml VirTis homogenizing flask using microblades

Characterization of Soluble RNA Polymerase

Employing the assay described in Experimental, using denatured calf thymus DNA as a template, the reaction was completed after 6–10 min If additional substrates (nucleoside triphosphates), DNA or enzyme were added to the assay after 10 min, little increase in activity was observed The reaction was linear with enzyme concentration up to 40 μg of protein The activity of RNA polymerase depended on the presence of each nucleoside triphosphate, added DNA and divalent metal ions (Table 2) The activity was completely inhibited by actinomycin-*D* and insensitive to rifampin As can be seen in Fig 1, the enzyme exhibited a sharp Mn^{2+} optimum of 1 25 mM with higher concentrations being extremely inhibitory An approximate concentration of 10 mM Mg^{2+} resulted in maximum enzyme activity, and higher concentrations (up to 35 mM) of Mg^{2+} were not inhibitory The Mn^{2+}/Mg^{2+} activity ratio (activity at optimum concentrations) was found to be approximately

[11] DUDA, C. T. and CHERRY, J H (1971) *Plant Physiol* **47**, 262

2·0 The optimum $(NH_4)_2SO_4$ concentration for the enzyme (50 mM) was quite low (2750 cpm incorporated/10 min) Higher concentrations were inhibitory, at 75 mM and 100 mM $(NH_4)_2SO_4$, incorporation was 2650 and 2520 cpm/10 min Without $(NH_4)_2SO_4$ and at 25 mM $(NH_4)_2SO_4$ activity was 2580 and 2700 cpm respectively

TABLE 2 COMPONENTS OF RNA POLYMERASE ASSAY

Assay components	% of control activity	Assay components	% of control activity	Assay components	% of control activity
Complete	100	−GTP	2	−Enzyme	0
−DNA	10	$-Mg^{2+}$	40	+Heated enzyme (60°, 10 min)	0
−ATP	3	$-Mn^{2+}$	80		
−CTP	1	$-Mg^{2+}/Mn^{2+}$	5	+Actinomycin D (200 μg/ml)	0
				+Rifampin (200 μg/ml)	100

Control activity (as described in Experimental) was 392 pmol of UMP incorporated per mg protein in 10 min

The enzyme had a slight preference for native calf thymus DNA as a template when compared to denatured DNA (Table 3) Native sugar beet DNA served as a template significantly better than denatured DNA Activities were very similar on native calf thymus DNA and native sugar beet DNA

TABLE 3 TEMPLATE SPECIFICITY FOR RNA POLYMERASE ACTIVITY

Template	pmol incorporated/ mg protein	Template	pmol incorporated/ mg/protein
Calf thymus (native)	350	Sugar beet (native)	368
Calf thymus (denatured)	325	Sugar beet (denatured)	246

Assays were as described in Experimental Calf thymus DNA was purchased from Sigma Co , and sugar beet DNA was extracted by a procedure which included ribonuclease and pronase treatment followed by hydroxyatite chromatography DNA was denatured at 100° for 5 min and cooled on ice

α-Amanitin Studies

Several workers[12,13] have shown that α-amanitin, a toxin extracted from the basidiomycete *Amanita phalloides*, specifically inhibits RNA polymerase II which is located in the nucleoplasm of eukaryotic cells RNA polymerase I, the nucleolar enzyme, has been shown to be insensitive to this toxin Employing the standard assay, maximum inhibition of the enzyme activity was observed at concentrations ranging from 0 01 to 0·05 μg/ml At the maximum inhibitory concentration (25 ng/ml), the polymerase activity was inhibited by 23 %

[12] LINDELL, T J , WEINBERGH, F , MORRIS, P W , ROEDER, R G and RUTTER, W J (1970) *Science* **170,** 447
[13] KEDINGER, C , NURET, P and CHAMBON, P (1971) *FEBS Letters* **15,** 169

It has been shown, however, that the various RNA polymerases have different salt and metal ion concentration optima [4,6,14] Therefore, further experiments employing α-amanitin were performed in the presence of optimum salt and metal ion concentrations specific for the polymerases as reported in other eukaryotic organisms The results of these experiments are presented in Table 4 If the values obtained in the presence of high salt concentration,

TABLE 4 EFFECTS OF α-AMANITIN ON RNA POLYMERASE ACTIVITY AT VARIOUS SALT AND METAL ION CONCENTRATIONS

Salt concentration	Metal ion	α-Amanitin (0 025 mg/ml)	Activity (pmol/mg/10 min)
—	Mg^{2+}, Mn^{2+}	—	393
Low	Mg^{2+}	+	288
High	Mn^{2+}	—	272
High	Mn^{2+}	+	216

$(NH_4)_2SO_4$ concentrations—Low = 50 mM, high = 300 mM Metal ion concentrations—Mg^{2+} = 10 mM, Mn^{2+} = 1 25 mM

Mn^{2+} and α-amanitin are subtracted from the activity when high salt concentration and Mn^{2+} only are present, the value obtained is an indication of the activity due to polymerase II (56 pmol/mg/10 min) Polymerase I activity is then assumed to be that activity observed in the presence of low salt concentrations, Mg^{2+} and α-amanitin (288 pmol/mg/10 min)

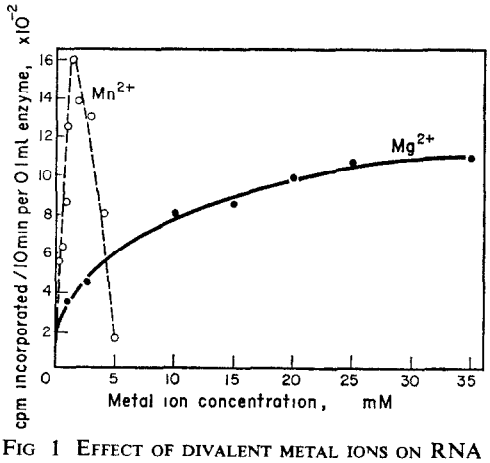

FIG 1 EFFECT OF DIVALENT METAL IONS ON RNA POLYMERASE ACTIVITY
Enzyme (40 μg protein in assay) was assayed using calf thymus DNA as template with only one metal ion present in the assay

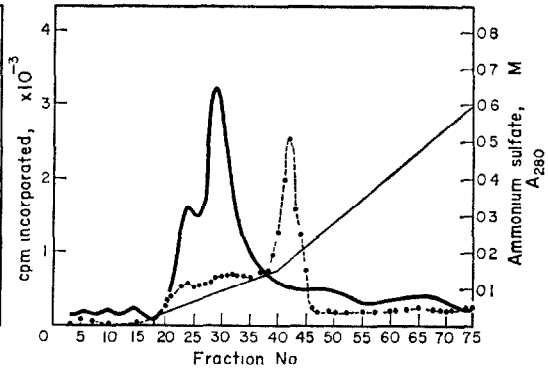

FIG 2 DEAE-SEPHADEX CHROMATOGRAPHY
Enzyme (2–3 mg proteins) was chromatographed as described in Experimental Fractions were assayed as described, employing denatured calf thymus DNA as template

If these assumptions are valid for this crude preparation, RNA polymerase I represents the major portion of the total activity The remaining activity may be attributable to RNA polymerase II and another nuclear RNA polymerase[4,6] or perhaps a polymerase from an organelle [9,15] α-Amanitin was observed to have little effect on the activity solubilized from

[14] BLATTI, S P, INCLES, C J, LINDALL, T J, MORRIS, P W, WEAVER, R F, WEINBERG, F and RUTTER, W J (1970) *Cold Spring Harbor Symp Quant Biol* **35**, 727
[15] TSAI, M, MICHAELIS, G and CRIDDLE, R S (1971) *Proc Natl Acad Sci U S* **68**, 473

unwashed tissue It should also be noted that in a 20-hr wash period the specific activity of the solubilized enzyme(s) increased from 2 4 pmol/min/mg of protein in unwashed tissue to 39·2 pmol/min/mg for tissue washed for 20 hr

DEAE-Sephadex Chromatography

To elucidate the possible presence of multiple polymerases indicated by the above inhibitor studies, the enzyme preparation was subjected to DEAE-Sephadex chromatography as described in the Experimental. The enzyme was eluted from the column in one major peak of activity at a salt concentration of 0 18 M (Fig 2) This major enzyme peak followed two major protein peaks which have recently been shown to contain DNA polymerase activities (data not presented)

DISCUSSION

The washing of sugar beet tissue has been shown to result in increased chromatin-bound RNA polymerase activity [11] When solubilized from chromatin of washed tissue and compared with the enzyme solubilized from chromatin isolated from unwashed beets, the specific activity of the 'washed' RNA polymerase was much higher It is difficult, based on these data, to determine whether this increase is due to increased synthesis of the enzyme or activation of the enzyme or both Unpublished data, in which cycloheximide was placed in the washing buffer at concentrations as high as 10 mg/ml, indicate that protein synthesis may not be necessary for the increase in RNA polymerase activity A quantitative measurement of the total activity per g fr wt is difficult due to the isolation technique employed to obtain chromatin Even though scraping of the pellets to remove the crude chromatin layer may vary, consistent results are obtained between experiments

The solubilization technique employed seems to be, in our experience, the best of those procedures attempted Because of apparent protein denaturation by temperature fluctuations and frothing, sonication and homogenization techniques were found to be unsuitable Some mechanical mixing is necessary, however, in that poor yields were observed if the chromatin was just allowed to incubate in the solubilization media, as has been employed with soybeans [16]

A comparison can be made between the activity of sugar beet RNA polymerase and that obtained from other organisms [4,6,14] The properties (salt and metal ion requirements, elution from DEAE-Sephadex) of the sugar beet enzyme compare quite closely with those of RNA polymerase I isolated from other systems Since the sugar beet enzyme is not fully purified, further studies may alter these data to some extent

It is interesting to note that γ-rays inhibit the activity of sugar beet soluble and chromatin-bound RNA polymerase [10,17] Since sugar beet RNA polymerase activity appears to be mostly enzyme I, it would be expected that ribosomal RNA synthesis would also be inhibited by radiation treatment As might be predicted, γ-rays inhibited the synthesis of both light and heavy ribosomal RNAs [17]

EXPERIMENTAL

Plant material and washing procedures Field grown sugar beet roots (stored at 4°) were sliced in 2–3 mm^3 sections, washed with sterile distilled H_2O and sterile PO_4^{2-} buffer, pH 6 5, as described previously [9] Flasks containing the tissue were shaken at 30° for the desired washing period

[16] HARDIN, J W and CHERRY, J H (1972) *Biochem Biophys Res Comm* **48**, 299
[17] DUNHAM, V L and CHERRY, J. H (1973) *Phytochemistry* **12**, 1891

Chromatin isolation, storage and assay Chromatin was isolated from washed or unwashed tissue by a modification of a procedure described by Huang and Bonner [18] Sugar beet tissue (900 g) was chilled to 4° and homogenized in 150 g lots with an equal vol of medium (125 mM sucrose, 100 mM Tris-HCl, pH 8 0, 100 mM $MgCl_2$, and 20 mM 2-mercaptoethanol) Dow Corning antifoam-*B* (6 drops) was added prior to homogenization After filtration through cheesecloth and Miracloth, the extract wes centrifuged at 8000 *g* for 20 min and the supernatant discarded The chromatin layer was scraped from the underlying starch and cellular debris, suspended in 10 ml of wash buffer (10 mM Tris-HCl, pH 8 0, 250 mM sucrose and 10 mM 2-mercaptoethanol) and centrifuged at 10 000 *g* for 20 min The chromatin layer was again scraped from the remaining pellet and resuspended in 3 ml of wash buffer The total chromatin preparation was divided in half, and layered over 20 ml of 2 M sucrose (containing 10 mM Tris-HCl, pH 8 0, and 10 mM 2-mercaptoethanol) in a SW 25 (Spinco) centrifuge tube After centrifugation at 20 000 rpm for 3 hr, the supernatant was removed by a syringe, the sides of the tubes carefully wiped dry and the chromatin pellets taken up in a total vol of 1 25 ml of 100 mM Tris-HCl (pH 8 0), and 10 mM 2-mercaptoethanol An equal vol of cold glycerol was added, mixed by vortexing, and the chromatin suspension was stored in liquid N_2 Chromatin-bound RNA polymerase activity was assayed as described previously [10] The standard assay contained 20 μmol of Tris-HCl (pH 8 0), 1 0 μmol $MgCl_2$, 0 25 μmol $MnCl_2$, 1 0 μmol of dithiothreitol, 0 2 μmol each of GTP, CTP, and ATP, 0 1 μmol of ^{3}H-UTP (specific radioactivity 17 4 Ci/mmol), and chromatin corresponding to 5–8 μg of DNA in a final vol of 0 4 ml The assay was performed at 35 for the desired time and stopped by the addition of 4 ml of cold 10% TCA (trichloroacetic acid) containing 1 mM pyrophosphate The ppts were collected on glass filters (Whatman GF/A), washed repeatedly with cold 5% TCA and the radioactivity determined with a scintillation spectrometer (Packard model 3375) When prepared and stored under the conditions described above, chromatin-bound RNA polymerase activity was quite stable For example, chromatin stored in liquid N_2 in the presence of 50% glycerol for as long as 3 weeks lost little activity

Solubilization of RNA polymerase from chromatin The enzyme was solubilized from the chromatin by combining chromatin from 900 g of sugar beet tissue (2 5 ml) with 1 ml of cold solubilization medium (40 μmol Tris-HCl, pH 8 0, 20 μmol dithiothreitol, 20 μmol $MgCl_2$ and 1 5 mmol $(NH_4)_2SO_4$ buffered at pH 8 0 with NH_4OH) The entire solution was stirred on ice for 2 hr, after which the solution was centrifuged in an SW39 (Spinco) rotor at 30 000 rpm for 1 hr The supernatant was collected and passed through a Sephadex G50 column (2 5 × 7 5 cm) which was previously washed with TGMED minus $MgCl_2$ (50 mM Tris-HCl, pH 7 9, 25% glycerol, 0 1 mM EDTA and 0 5 mM dithiothreitol[6]) The enzyme was eluted from the column with the above buffer The eluate was monitored at 280 nm and the peak tubes combined This procedure served to remove the inhibitory levels of $(NH_4)_2SO_4$ present in the solubilization medium The enzyme was then assayed and characterized or subjected to DEAE-Sephadex chromatography

DEAE-Sephadex chromatography A sample of the enzyme preparation described above (2–3 mg protein) was chromatographed on a 0 9 × 14 cm DEAE-Sephadex A25 column The column was eluted with 2 gradients (a) 0 025–0 15 M $(NH_4)_2SO_4$ in TGMED, 20 ml total, (b) 0 15–0 6 M $(NH_4)_2SO_4$ in TGMED, 40 ml total

RNA polymerase assay The soluble enzyme was typically assayed as follows the assay contained 20 μmol Tris-HCl, pH 8 0, 4 μmol $MgCl_2$, 0 5 μmol $MnCl_2$, 10 μmol of dithiothreitol, 0 2 μmol each of GTP, CTP, and ATP, 0 1 μmol of ^{3}H-UTP (specific radioactivity 17 4 Ci/mmol), 20 μg DNA and 0 05 ml enzyme (corresponding to *ca* 40 μg protein) The reaction was initiated by the addition of enzyme, run for 10 min at 37° and stopped with the addition of the following cold solutions in sequence 0 5 ml of 100 mM sodium pyrophosphate, pH 7 0, 0 1 ml of 5% sodium dodecyl sulfate and (while mixing) 2 ml of 10% TCA containing 40 mM sodium pyrophosphate Following 15 min storage at 0°, the ppts were collected on glass filters, washed with 5% TCA, and the radioactivity determined as described above All chemicals used were reagent grade ^{3}H-UTP was purchased from Schwarz Bioresearch, Orangeburg, N Y α-Amanitin was purchased from Henley & Company, New York, and rifampin from CIBA Pharmaceutical Company, Summit, New Jersey Protein determination was by the Lowry[19] method and DNA by the diphenylamine colorimetric procedure of Burton [20]

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