THE SEPARATION OF RNA POLYMERASES I AND II ACHIEVED BY FRACTIONATION OF PLANT CHROMATIN

C.Y. LIN, T.J. GUILFOYLE, Y.M. CHEN, R.T. NAGAO AND J.L. KEY

Department of Botany, University of Georgia, Athens, Georgia 30602

Received July 8,1974

SUMMARY

Chromatin-bound RNA polymerase I and II from soybean hypocotyl can be separated by differential centrifugation. While all detectable RNA polymerase I is pelleted in association with chromatin, nearly all of the RNA polymerase II activity is not pelleted even at high speeds. Substitution of pH 6 for pH 8 isolation medium results in several-fold greater recovery of chromatin-bound RNA polymerase II.

INTRODUCTION

The direct isolation of plant chromatin (1) is routinely used to study in vitro RNA synthesis (2,3,4) since plant nuclei are generally quite difficult to isolate in sufficient quantity for extensive biochemical work. This procedure employs homogenization in a medium buffered at pH 8 and centrifugation at low g forces (e.g. 4,000) to isolate a crude chromatin which is subsequently purified by centrifugation through dense sucrose (e.g. 1.8M). The procedure has been reported to result in a chromatin preparation possessing little or no α-amanitin sensitive RNA polymerase activity (5). This result implies that the chromatin isolated by this procedure contains primarily RNA polymerase I or if RNA polymerase II is present, it fails to be expressed on the chromatin. Accordingly we have conducted a series of experiments designed to investigate the distribution of RNA polymerase activities in the tissue homogenate utilizing different methods of chromatin preparation. The RNA polymerases have been solubilized and fractionated to detect whether the α-amanitin sensitive enzyme is suppressed on the chromatin. Our results show that the chromatin-bound α-amanitin insensitive enzyme is RNA polymerase I and that chromatin prepared by the above referenced method contains only trace amounts of RNA polymerase II. Some RNA polymerase II activity can be recovered in lighter chromatin fractions; however, about 90% of the RNA polymerase II activity

remains in a high speed supernatant and is not recovered in pelleted chromatin fractions. Isolation of chromatin at acid pH does result in higher yields of chromatin-bound RNA polymerase II, but still a relatively small % of the total.

MATERIALS AND METHODS

Chromatin was isolated from mature hypocotyl of 4-day-old, etiolated soybean (Glycine max, variety Wayne) seedlings which were germinated at 28°C in moist vermiculite. Tissue sections were homogenized in 250 mM sucrose, 1mM $MgCl_2$, 10 mM 2-mercaptoethanol, and either 50 mM Tris (pH 8.0) or 50 mM MES (2[N-Morpholino]ethane sulfonic acid) (pH 6.0) for 1 minute at setting #6 with a Willems Polytron Model PT 20-ST (Brinkmann Instruments, Inc.). Although the Polytron was used for routine chromatin isolation, homogenization with a Waring blender, Virtis homogenizer, and mortar and pestle resulted in similar chromatin preparations. Homogenization and all subsequent procedures were conducted at 0-4°C. The homogenate was filtered through 4 layers of cheesecloth and a layer of miracloth (Calbiochem). The filtrate was centrifuged at 4000 Xg for 30 minutes and the pellet (Heavy Chromatin) was collected. The supernatant was recentrifuged at 17,000 Xg for 10 minutes and a second pellet (Light Chromatin A) was collected. The supernatant from this fraction was centrifuged at 25,000 rpm for 30 minutes in a SW 27 rotor with a Beckman L2-65 ultracentrifuge. A third pellet (Light Chromatin B) and the supernatant (Soluble Phase) were collected. Pelleted chromatin fractions were not purified through dense sucrose since this results in loss of DNA and isolation of a chromatin which has only traces of α-amanitin sensitive activity (6). Instead crude chromatin pellets were washed with 1% Triton X-100 plus buffer, and then two times with buffer minus Triton. This procedure did not result in detectable loss of either DNA or RNA polymerase activity, but a considerable amount of protein was removed from the chromatin pellets.

The buffer solution used to solubilize RNA polymerase activities was 50mM Tris (pH 8.0), 10 mM dithiothreitol, 5 mM $MgCl_2$, 0.1 mM EDTA, 25% glycerol, and 0.5 mM phenylmethylsufonylfluoride containing 500 mM $(NH_4)_2SO_4$. The chromatin suspension was stirred for 5 hours at 0°C followed by sonication for

twenty seconds (3X) with a microtip probe (Lab-Line Instrument, Inc.). Residual chromatin was pelleted at 40,000 rpm in a T65 rotor. Soluble polymerase was precipitated with solid $(NH_4)_2SO_4$ (7), dialyzed against the above buffer containing 50 mM $(NH_4)_2SO_4$, and fractionated on DEAE cellulose.

RNA polymerase was assayed in 250 µl reaction mixtures containing 50 mM Tris (pH 8.0), 5% glycerol, 10 mM dithiothreitol, 0.4 mM ATP, CTP, GTP, and 0.01 mM [^{3}H]UTP (2.5 µC) and other additions as described in the tables and figures.

RESULTS

Soybean chromatin can be separated into several distinct fractions with respect to RNA polymerase activities by differential centrifugation (Table I). Heavy chromatin contains little α-amanitin sensitive (RNA polymerase II) activity when isolated at alkaline pH. However, the amount of heavy chromatin-

Table I. Chromatin-bound RNA Polymerase Activities

	pH 8		pH 6	
Chromatin Fraction	PI	PII	PI	PII
	pmoles [^{3}H]UTP incorp./g fresh wt.			
Heavy Chromatin	8.1	2.2	10.9	19.0
Light Chromatin (A)	0.4	0.9	0.8	2.4
Light Chromatin (B)	0.6	1.8	1.0	1.9

Chromatin-bound polymerase I (PI) (α-amanitin insensitive) activity was assayed optimally with 1 mM $MnCl_2$ and 200 mM KCl in the presence of 4 µg/ml α-amanitin. Polymerase II (PII) (α-amanitin-sensitive) activity was assayed optimally with 10 mM $MgCl_2$ and 400 mM KCl. Polymerase II activity was determined as the difference in pmoles incorporated in the absence of α-amanitin and pmoles incorporated in the presence of 4 µg/ml α-amanitin. Reactions were terminated after 20 minutes.

bound RNA polymerase II recovered is increased several-fold when chromatin is isolated at pH 6. The recovery of α-amanitin insensitive chromatin activity (RNA polymerase I) is not significantly affected by the pH of the isolation medium. Practically all RNA polymerase I activity is recovered in the heavy chromatin fraction independent of the pH. At pH 8, about 60 percent of the chromatin-bound RNA polymerase II is recovered in light chromatin fractions (A & B) while at pH 6 about 80% of chromatin-bound polymerase II is recovered in the heavy chromatin fraction. The overall recovery of chromatin-bound RNA polymerase II is nearly 5-fold greater at pH 6 than at pH 8. Although nearly all of the detectable RNA polymerase I is recoverable in a chromatin-bound condition, only about 10% of the total RNA polymerase II is recovered in chromatin with about 90% appearing to be free in the supernatant. The distribution of RNA polymerase I and II and DNA in the various chromatin and soluble fractions is shown in Table II.

The distribution of RNA polymerase I and II activities from chromatin, soluble phase, and total tissue is further delineated by the DEAE cellulose profiles shown in Figure 1. These distributions substantially agree with the values of Table II based on α-amanitin sensitivity. At pH 8, heavy chromatin activity is almost totally due to RNA polymerase I (A) while light chromatin has

Table II. Distribution of RNA Polymerase I and II and DNA.

Enzyme Source	Recoverable DNA		Total Polymerase I		Total Polymerase II	
	pH 8	pH 6	pH 8	pH 6	pH 8	pH 6
	percent					
Heavy Chromatin	40	80	90	90	2	10
Light Chromatin (A & B)	40	10	10	10	5	2
Soluble Phase	20	10	0	0	93	88

Assay conditions are described in Table I. Soluble phase was assayed after step-off elution from DEAE cellulose.

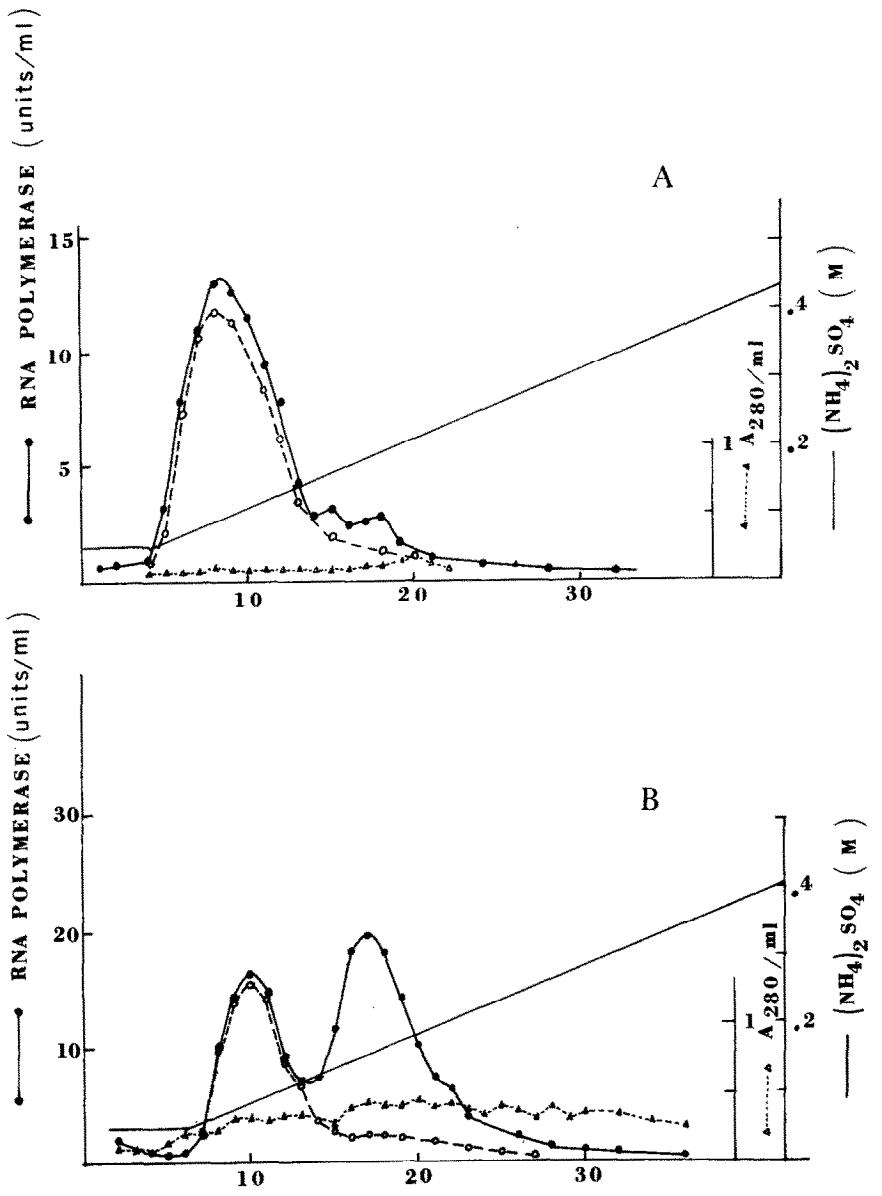
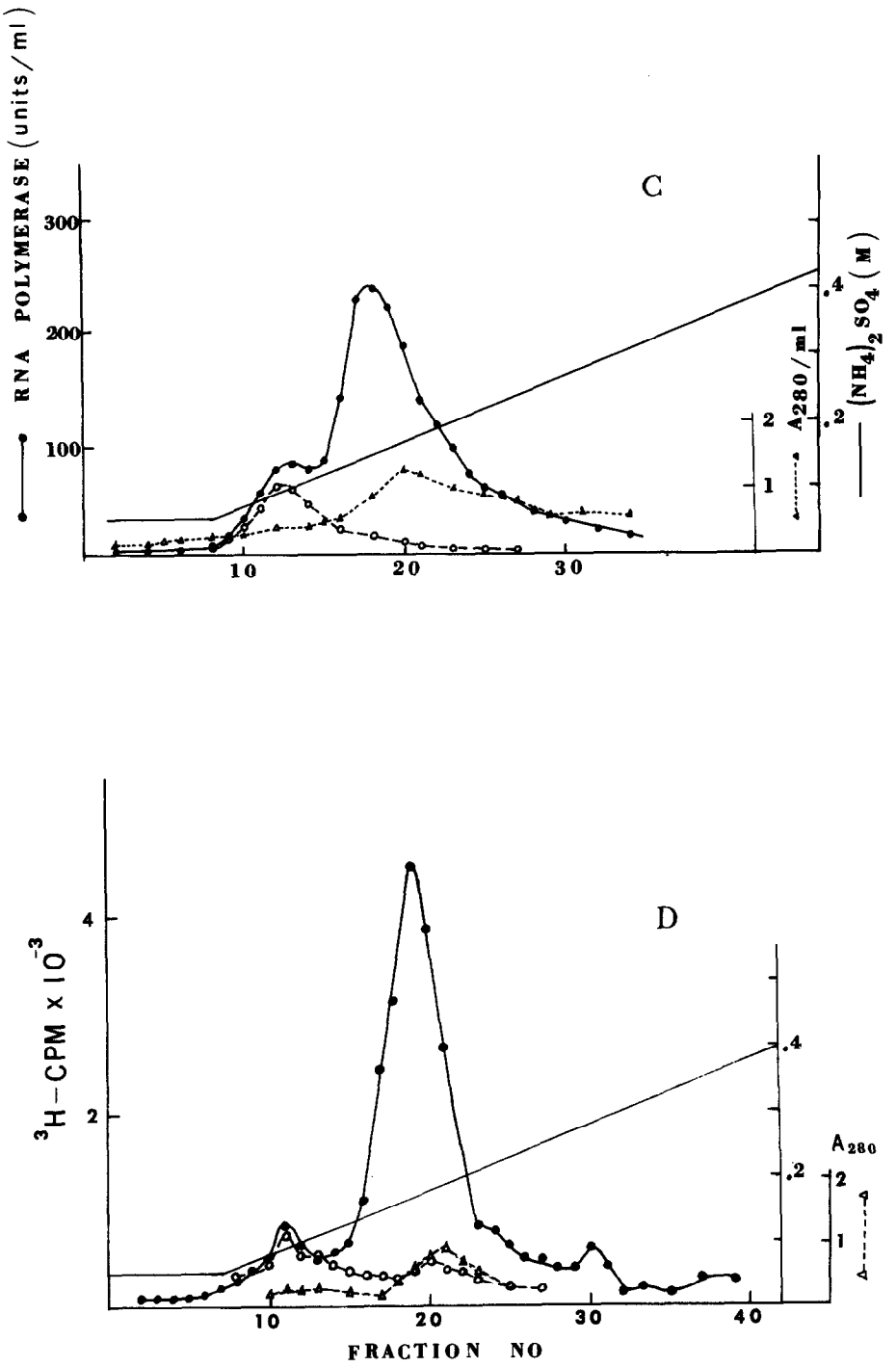
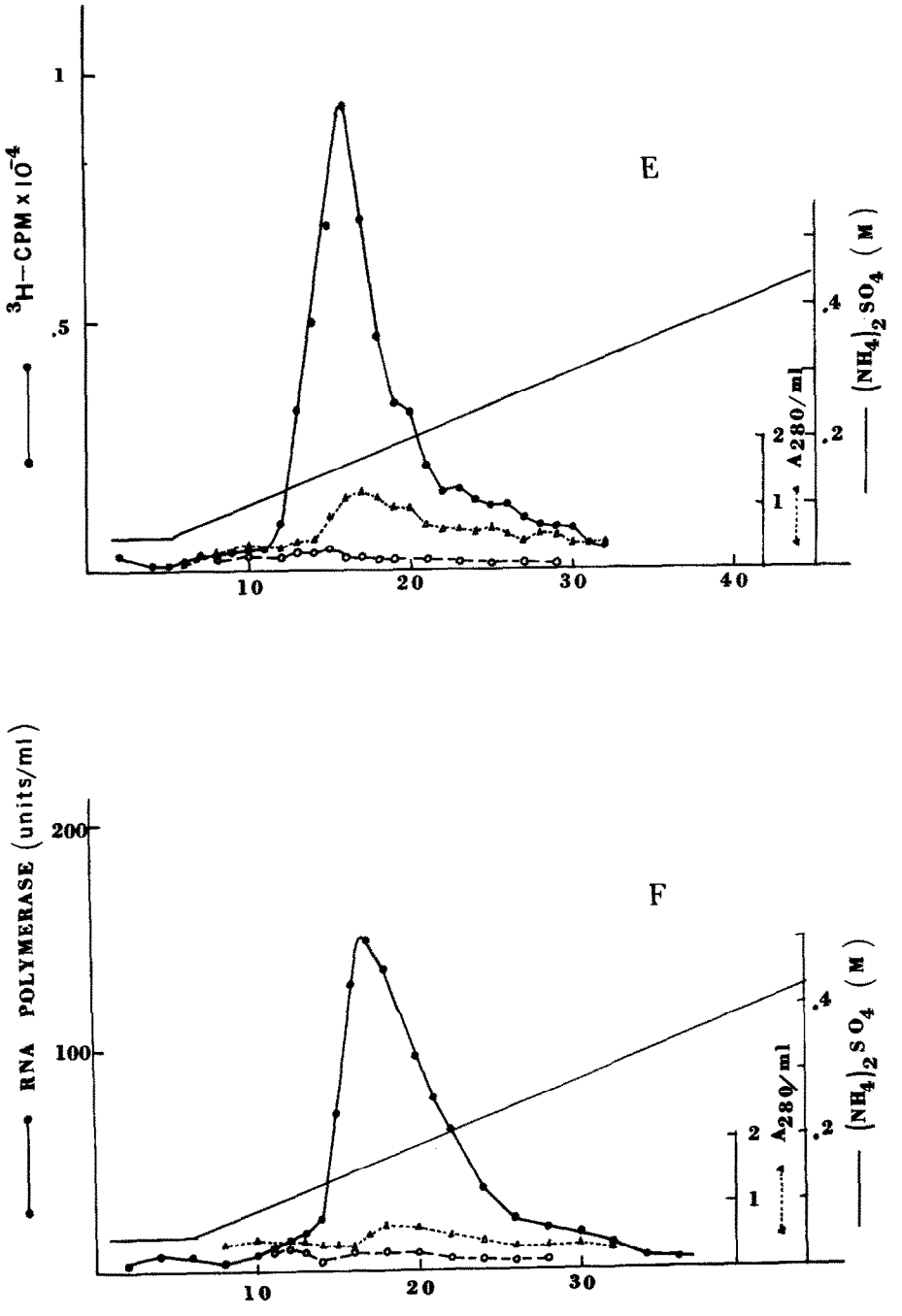


Figure 1. DEAE cellulose profiles of RNA polymerase activity. RNA polymerase assays included 20 µg denatured calf thymus DNA, 1 mM $MnCl_2$ and 5 mM $MgCl_2$. Figures 1 D and E were assayed in the presence of 0.3 µM UTP while all others included 0.01 mM UTP. 1 Unit = 1 pmole incorp./20 min. at 28°C. A: Heavy chromatin pH 8.0; B: Light chromatin (A & B) pH 8.0; C: Heavy chromatin pH 6.0; D: Total tissue pH 8.0; E: Soluble phase pH 8.0; F: Soluble phase pH 6.0; Solid circles: No α-amanitin; Open circles: plus α-amanitin (4 µg/ml).


C
RNA POLYMERASE (units/ml)
300
200
100
10
20
30
A280/ml
2
1
.4
.2
$(NH_4)_2SO_4$ (M)
D
^3H-CPM x 10^{-3}
4
2
10
20
30
40
FRACTION NO
A_{280}
2
1
.4
.2


E
^{3}H–CPM x10^{-4}
1
.5
10
20
30
40
A280/ml
2
1
.4
.2
$(NH_4)_2SO_4$ (M)
F
RNA POLYMERASE (units/ml)
200
100
10
20
30
A280/ml
2
1
.4
.2
$(NH_4)_2SO_4$ (M)

a greater amount of RNA polymerase II (B). Heavy chromatin isolated at pH 6.0 (C) has an RNA polymerase profile significantly enriched in RNA polymerase II when compared to heavy chromatin isolated at pH 8 (A). The fractionation of whole tissue (D) gives an RNA polymerase I : II ratio of 1 : 20. The supernatant or soluble phase remaining after light chromatin is pelleted, contains the major portion of RNA polymerase II (E and F) while no polymerase I is detectable in this fraction.

DISCUSSION

We have shown that under alkaline conditions (and to a somewhat lesser degree under acidic conditions) the direct isolation of chromatin may result in preferential sedimentation of a select fraction of nucleoprotein which is rich in RNA polymerase I. Little chromatin-bound RNA polymerase II is sedimented at alkaline pH. Failure to detect higher levels of chromatin-bound RNA polymerase II is not due to its suppression or inactivity on the chromatin template since the relative proportions of RNA polymerase I and II are maintained when the enzymes are solubilized, fractionated, and made dependent on an exogenous source of DNA.

Although this laboratory has taken advantage of alkaline separation of RNA polymerase I and II as a first step in their purification, the isolation of plant chromatin under these conditions must be handled with caution. The augmentation of RNA polymerase activity in response to plant hormones is routinely studied on heavy chromatin fractions isolated at alkaline pH; this may preclude any observation of a hormonally-induced change in RNA polymerase II activity. This possibility will be discussed in a forthcoming publication, dealing with auxin-induced RNA polymerase acitivity in soybean.

ACKNOWLEDGEMENTS

This work was supported by Public Health Service Research Grant Ca 11624 from the National Cancer Institute.

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