Ribonucleic Acid Polymerase Activities in Jerusalem Artichoke Tissue

By JOHN R. GORE and JOHN INGLE

*Department of Botany, University of Edinburgh, King's Buildings, Mayfield Road,
Edinburgh EH9 3JH, U.K.*

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1. Artichoke tuber tissue contained RNA polymerase activity bound to the chromatin and in the supernatant after chromatin sedimentation. 2. The activity in the supernatant, the soluble polymerase, was fractionated into polymerases I and II by DEAE-cellulose chromatography, and the properties of each activity were determined. 3. The proportions of chromatin-bound and soluble activities varied with growth of the tissue, and there was a correlation between chromatin-bound activity and RNA accumulation. 4. The properties of the solubilized chromatin activity were compared with those of the soluble activity, and the relationship between these two activities is discussed.

In eukaryote tissues RNA polymerase is normally associated with chromatin when the latter is prepared under low-salt conditions. In pea (Huang *et al.*, 1961; McComb *et al.*, 1970), soya bean (O'Brien *et al.*, 1968; Holm *et al.*, 1970), hazel (Jarvis *et al.*, 1968), cucumber (Johnson & Purves, 1970) and sugar beet (Duda & Cherry, 1971), the bulk of the RNA polymerase is associated with the chromatin, but in maize, under comparable isolation conditions, less than 1% is associated with chromatin, the remaining 99% of the polymerase activity being in the supernatant after chromatin sedimentation, and requiring exogenous DNA for activity (Stout & Mans, 1967). The solubility of maize RNA polymerase has facilitated further studies and this activity has been resolved into two components, polymerases I and II, by DEAE-cellulose chromatography (Strain *et al.*, 1971). The properties of these two activities are similar in most respects to the two polymerases solubilized from rat liver nuclei (Roeder & Rutter, 1969). Both chromatin-bound and soluble RNA polymerase activities appear to be present in wheat (Polya & Jagendorf, 1971a) and sugar beet (Duda & Cherry, 1971). In the present paper we show that artichoke tuber tissue also contains chromatin-bound and soluble RNA polymerase activities. The properties of these activities have been studied, and the relationship between them has been examined in tissue cultured under various physiological conditions resulting in very different patterns of RNA synthesis.

Materials and Methods

The tissue used in this study was isolated from tubers of a single clone of Jerusalem Artichoke (*Helianthus tuberosus* L, var. Bunyards Round). The tubers were harvested in November, by which time they had achieved maximum size, and stored under damp sand

at 4°C until use. Tuber tissue was used directly for the main characterization of the polymerase activities.

For the investigation of polymerase activity under different growth conditions, cylindrical explants (2 mm diam. × 2.4 mm long) were aseptically removed from surface-sterilized tubers and incubated in the dark in medium with and without 2,4-dichlorophenoxyacetic acid, the growth substance required for cell division in this system (Yeoman *et al.*, 1965).

Preparation of chromatin-bound and soluble polymerase activities

For characterization of the polymerase activities scrubbed artichoke tubers were sliced into cold homogenizing medium [50 mM-Tris-HCl, pH 8.0, 50 mM-2-mercaptoethanol, 5 mM-MgCl₂, 5% (v/v) glycerol and 1 mg of polyvinylpyrrolidone/ml] and the tissue was homogenized for 5 s at full speed in the Polytron homogenizer. The homogenate was filtered through one layer of Miracloth and centrifuged at 10000g for 10 min at 0°C. The gelatinous chromatin pellet was scraped from an underlying layer of dark-coloured material and resuspended in a glass-in-glass homogenizer (Kontes C) in 10 ml of wash medium (50 mM-Tris-HCl, pH 8.0, and 50 mM-2-mercaptoethanol). The chromatin was collected by centrifugation at 10000g for 10 min, suspended in 2 ml of wash medium and layered on to 19 ml of 75% (v/v) glycerol containing 50 mM-2-mercaptoethanol. The purified chromatin, recovered by centrifugation at 40000g in a SW 30 MSE rotor for 2 h 45 min at 4°C, was suspended in 2 ml of 0.15 M-Tris-HCl (pH 8.0)–0.5 mM-dithiothreitol and assayed for RNA polymerase activity.

The supernatant from the initial 10000g centrifugation was used for the preparation of the soluble polymerase activity. Precipitation with (NH₄)₂SO₄ (35–65% satd.) resulted in a fivefold purification. The

precipitate was dissolved in standard buffer (5% glycerol, 50mM-Tris-HCl, pH8.0, 0.5mM-dithiothreitol, 5mM-MgCl₂), desalted on a Bio-Gel P4 column (15cm×1cm diam.) and fractionated on a DEAE-cellulose column (10cm×1cm diam.), by using a 60ml linear gradient from 0 to 0.5M-(NH₄)₂SO₄ in standard buffer.

These methods were adapted for use with the smaller amount of tissue available when studying polymerase activity in the cultured explants. Explants (25) were homogenized by six strokes in a glass-in-glass Kontes homogenizer in 1ml of homogenizing medium, and the homogenate filtered through a pre-wetted Miracloth filter (2cm diam). The homogenizer was rinsed with 0.5ml of homogenizing medium, and the combined filtrates were centrifuged at 10000g for 10min at 0°C. The supernatant was assayed directly for soluble polymerase activity. The chromatin pellet was washed once with standard buffer, collected by centrifugation and suspended in 1ml of standard buffer for assay of chromatin-bound activity.

RNA polymerase assay

The assay used was based on that of O'Brien *et al.* (1968), which measures the incorporation of [³H]UTP into a trichloroacetic acid-insoluble product. Portions (0.2ml) of the RNA polymerase preparation were incubated for 10min at 30°C in a final volume of 0.4ml containing 1.0μmol of MgCl₂, 0.5 μmol of MnCl₂, 0.2μmol each of ATP, CTP and GTP, 10μmol of Tris-HCl, pH8.0, 0.2μmol of dithiothreitol and 2μCi of [³H]UTP (specific radioactivity 1.4Ci/mmol). A DNA template was included in the assay of the soluble RNA polymerase (50μg of denatured calf thymus DNA/assay) but not in the assay of chromatin-bound activity. The reaction was terminated by the addition of 2.0ml of ice-cold 5% (w/v) trichloroacetic acid containing 0.01M-tetrasodium pyrophosphate, and the contents of the tube were then transferred to a GF/C 2.1cm disc and washed with 50ml of cold 5% trichloroacetic acid plus pyrophosphate, followed by 5ml of 95% (v/v) ethanol. The filters were finally dried and counted for radioactivity by liquid-scintillation counting in 4% (w/v) 2-(4-t-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole in toluene. Both the soluble and chromatin-bound activities were linear with time over this time-period.

Results

Properties of the chromatin-bound and soluble polymerase activities (see Table 1)

The incorporation of [³H]UTP (or [³H]CTP) by either activity was dependent on the presence of other nucleoside triphosphates. The major difference between the two activities was the complete depen-

dence of the soluble activity on exogenous denatured DNA. The chromatin-bound activity was stimulated a little by exogenous denatured DNA, but was inhibited by native DNA, suggesting that there may be some competition between endogenous and exogenous DNA for the polymerase or other chromatin proteins. Digestion of the template, either endogenous or exogenous, with DNAase* resulted in complete loss of activity. Addition of KCl stimulated the activity of the chromatin-bound polymerase up to 150% at 0.25M, whereas soluble activity was increasingly inhibited by increasing salt concentration. The concentrations of Mg²⁺ or Mn²⁺ necessary for optimal activity were higher for the chromatin-bound activity (2.5mM) than for the soluble (1.25mM). Further, whereas Mg²⁺ by itself stimulated activity of the chromatin-bound activity above that of the control (containing 2.5mM-Mg²⁺, 0.625mM-Mn²⁺), and activity in the presence of Mn²⁺ alone was equal to that of the control, neither bivalent cation alone stimulated activity of the soluble polymerase to the control value. Soluble polymerase activity was 50% inhibited by the fungal toxin α-amanitin, compared with 20% inhibition of the chromatin-bound activity. The chromatin-bound and soluble RNA polymerase activities therefore differ in their DNA dependency, their response to salt and to bivalent cations and in their sensitivity towards α-amanitin.

Table 1. *Properties of chromatin-bound and soluble RNA polymerase activities*

The values are the average of replicate assays and are expressed as a percentage of the control assay, as detailed in the Materials and Methods section.

Conditions of assay	RNA polymerase activity (% of control)	
	Chromatin-bound	Soluble
-GTP	11	20
Denatured DNA (50μg)	138	100†
-DNA	100†	3†
Native DNA (50μg)	37	6
DNAase (10μg/ml, 10min, 30°C)	0	0
KCl (0.25M)	150	25
1.25mM-Mg ²⁺ , -Mn ²⁺	130	70
2.5mM-Mg ²⁺ , -Mn ²⁺	165	70
1.25mM-Mn ²⁺ , -Mg ²⁺	70	60
2.5mM-Mn ²⁺ , -Mg ²⁺	100	35
α-Amanitin (0.1μg/ml)	80	50

† Control of chromatin-bound and soluble assays.

* Abbreviation: DNAase, deoxyribonuclease.

Quantitative distribution of chromatin-bound and soluble polymerase activities

Determination of the quantitative distribution of the polymerase activities was facilitated by their different template and ionic requirements. Contamination of the soluble activity by chromatin was shown to be negligible by assaying the supernatant preparation in the absence of DNA and the presence of 0.25M-KCl, conditions optimal for chromatin activity. The extent of contamination of the chromatin preparation by soluble activity was not easily determined, but its contribution to the chromatin activity was minimized by the conditions of assay, 0.25M-KCl and no exogenous template. The total activities recovered, determined under conditions optimal for each activity, indicated that 20% of the total polymerase activity was associated with the chromatin in the fresh artichoke tuber.

Solubilization of the chromatin-bound polymerase activity

Although the properties of the chromatin-bound activity differed from those of the soluble preparation, the differences may result from the association of the enzyme with the chromatin rather than reflect inherent differences between the two enzyme activities. Attempts were therefore made to solubilize the chromatin-bound activity to allow a more meaningful comparison to be made. Omission of Mg^{2+} from the resuspension buffer did not result in any solubilization although the activity of the chromatin was significantly decreased, but the addition of EDTA did result in 40% of the activity being recovered in the supernatant, together with an increase in the total activity recovered (Table 2). Treatment of the chromatin with 0.3M-KCl resulted in solubilization of 60% of the activity, which was increased to 70% by additional sonication. Digestion of the DNA of the chromatin with DNAase resulted in 60% solubilization, but the recovery of total activity after the treatment was only 39%.

The dependence of the solubilized activity on exogenous DNA was complicated by the presence in the supernatant fraction of endogenous DNA, which could not be quantitatively removed without concomitant loss of polymerase activity. The response of the solubilized activity towards salt, which apart from the DNA requirement was the most characteristic difference between the soluble and the chromatin-bound polymerase activities, was more readily determined. All the solubilized activities were stimulated by KCl to the same extent as the initial chromatin-bound polymerase. This salt stimulation suggested that the polymerase activity solubilized from the chromatin differed from the soluble activity, although the possibility that the solubilized activity merely represented small fragments of chromatin, not

Table 2. *Solubilization of chromatin-bound polymerase*

Purified chromatin was incubated with (a) standard buffer (5% glycerol, 5mM- Mg^{2+} , 0.5mM-dithiothreitol, 50mM-Tris-HCl, pH8.0), (b) standard buffer minus Mg^{2+} , (c) standard buffer minus Mg^{2+} plus 10mM-EDTA, (d) standard buffer plus 0.3M-KCl, (e) standard buffer plus 0.3M-KCl followed by sonication for 4×30s at maximum ampage in the MSE Sonicator, (f) ribonuclease-free DNAase (10μg/ml). Treatments were for 90min at 4°C in Expts. 1 and 3, and for 30min at 4°C in Expt. 2. After treatment the chromatin was collected by centrifugation at 10000g for 10min and the pellet and supernatant were assayed for polymerase activity in the presence of 0.25M-KCl. All values are the average of replicate determinations. The supernatant activity assayed in the absence of KCl is shown in parentheses.

Treatment	Total activity (c.p.m. incorporated/10 min)		Solubilization (%)
	Chromatin	Supernatant	
Expt. 1			
(a) Control	8400	0	0
(b) -Mg ²⁺	5200	0	0
(c) +EDTA	6400	4380 (2850)	41
Expt. 2			
(a) Control	15000	0	0
(d) +0.3 M-KCl	5743	9205 (5750)	61
(e) +0.3 M-KCl +sonication	4300	10900 (7600)	72
Expt. 3			
(a) Control	50000	0	0
(f) DNAase	7400	12000 (9000)	62

sedimented at 10000g, could not be ruled out. The solubilized preparation was subjected to further purification by DEAE-cellulose chromatography under conditions successfully used for the fractionation of the soluble polymerase activity, but no activity was bound to the column.

Fractionation of the soluble polymerase activity

Fractionation of the soluble polymerase activity, after $(NH_4)_2SO_4$ precipitation and desalting, by DEAE-cellulose chromatography, resolved two peaks of activity eluted at 0.125M- and 0.3M- $(NH_4)_2SO_4$, termed polymerases I and II respectively (Fig. 1a). When polymerase I and II activities were collected, desalted and rechromatographed on DEAE-cellulose they re-ran as single peaks, eluted at 0.12M- and 0.3M- $(NH_4)_2SO_4$ respectively.

The two activities differed in their requirements for bivalent cations (Table 3). Whereas either Mn^{2+} or Mg^{2+} alone could support full activity of polymerase I, compared with the control assay containing 1.25mM- Mn^{2+} and 2.5mM- Mg^{2+} , polymerase II required only

Fig. 1. Fractionation of soluble polymerase activity

The soluble polymerase preparation was purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation, desalted on a Bio-Gel P4 column and loaded on a column of DEAE-cellulose DE32. The column was washed with standard buffer (5% glycerol, 50 mM-Tris-HCl, pH 8.0, 0.5 mM-dithiothreitol, 5 mM- MgCl_2) until the E_{280} was zero, and then eluted with a linear gradient of $(\text{NH}_4)_2\text{SO}_4$ (0–0.5 M in standard buffer; ----). The E_{280} of the eluate was continuously recorded (—) and samples of the 2 ml fractions were assayed for RNA polymerase activity (histogram) with denatured DNA as template (a). The polymerase II peak from a similar column was assayed by using both denatured (—) and native (····) DNA (b).

Mn^{2+} for the full control activity. Both the activities were inhibited by $(\text{NH}_4)_2\text{SO}_4$, polymerase II being more sensitive to low concentrations of the salt. Neither polymerase activity was specifically inhibited by rifampicin, but α -amanitin very specifically inhibited polymerase II, giving 0 and 90% inhibition of polymerases I and II respectively at 0.1 $\mu\text{g}/\text{ml}$. This differential sensitivity of the two activities towards α -amanitin indicates that the total soluble polymerase,

which is 50% inhibited by α -amanitin (Table 1) is made up of equal activities of polymerases I and II, an interpretation which is supported by the DEAE-cellulose fractionations. Both polymerase activities preferred denatured to native DNA for template, although there was considerable polymerase II activity on native DNA, particularly with high DNA concentrations in the assay. Since the template specificity of the enzyme may be affected by bivalent cation content and concentration, polymerase II was assayed on both denatured and native DNA with a range of bivalent cation concentrations. In the presence of 2.5 mM- Mg^{2+} and 0–5 mM- Mn^{2+} , the relative efficiency of polymerase II on native and denatured DNA was not changed, indicating that the $\text{Mg}^{2+}/\text{Mn}^{2+}$ ratio was not important in controlling the template specificity of this fraction.

The existence of multiple polymerases in peak II, with different template requirements was not indicated from the assay of peak II on both denatured and native DNA (Fig. 1b).

Distribution of polymerase activity during culture of artichoke tissue

DNA synthesis and cell division of the artichoke tissue was dependent on the presence of 2,4-dichlorophenoxyacetic acid in the incubation medium (Fig. 2). There was an initial increase in rRNA content independent of the presence of auxin. However, in the absence of auxin the rRNA content of the explant decreased after about 20 h back to the starting value, whereas accumulation of rRNA continued in the presence of auxin, reaching a plateau after 50 h. The changes in chromatin-bound polymerase activity were similar to those of RNA

Table 3. Properties of soluble polymerase activities I and II

Polymerase peaks I and II from a DEAE-cellulose column were desalted by passage through Bio-Gel P4 columns equilibrated with standard buffer minus Mg^{2+} . The preparations were assayed under conditions described below, and replicate activities expressed as a percentage of the control assay.

Conditions of assay	Polymerase activity (% of control)	
	I	II
1.25 mM- Mg^{2+} , – Mn^{2+}	85	45
2.5 mM- Mg^{2+} , – Mn^{2+}	100	50
1.25 mM- Mn^{2+} , – Mg^{2+}	120	95
2.5 mM- Mn^{2+} , – Mg^{2+}	115	55
$(\text{NH}_4)_2\text{SO}_4$ (0.2 M)	85	45
(0.8 M)	10	35
Rifampicin (200 $\mu\text{g}/\text{ml}$)	94	98
α -Amanitin (0.1 $\mu\text{g}/\text{ml}$)	100	12
Native DNA (10 $\mu\text{g}/\text{assay}$)	36	43
(50 $\mu\text{g}/\text{assay}$)	36	64

Fig. 2. RNA and DNA content during the culture of artichoke explants

Explants were cultured in the presence (—) and absence (---) of 2,4-dichlorophenoxyacetic acid. Ribosomal RNA ($\blacktriangle$, $\triangle$) and DNA ($\blacktriangledown$, $\triangledown$) contents were estimated from the areas of the relevant peaks from scans of polyacrylamide-gel fractionations of total nucleic acid preparations (Ingle, 1968) and expressed on an explant basis.

accumulation, with an initial increase which was independent of the growth conditions, followed by divergence of the cultures with and without 2,4-dichlorophenoxyacetic acid (Fig. 3a). Soluble polymerase activity did not show comparable increases during the initial 20h of culture (Fig. 3b), although there was a 50% increase in activity in the 2,4-dichlorophenoxyacetic acid-treated culture between 20 and 80h, during which period activity in the culture without 2,4-dichlorophenoxyacetic acid remained constant at a value lower than that of the fresh tuber tissue. Total polymerase activity in the dividing tissue therefore increased just less than twofold, the majority of the increase being due to the chromatin-bound activity, which increased from 30% of the total polymerase activity in the fresh tissue to 50% after 30h culture and then decreased to 30% by the end of the 80h period (Fig. 3c). Tissue cultured in the absence of 2,4-dichlorophenoxyacetic acid resulted in a similar initial increase in chromatin-bound activity up to 50% of the total, which then decreased to about 10% under conditions of no RNA accumulation.

Fig. 3. Changes in chromatin-bound and soluble polymerase activities during the culture of artichoke explants

Explants were cultured in the presence (—) and absence (---) of 2,4-dichlorophenoxyacetic acid. The average of replicates of chromatin-bound (a) and soluble (b) activities are expressed as pmol of UMP incorporated/10min per 100 explants. (c) The chromatin-bound activity is expressed as a percentage of the total (chromatin-bound plus soluble) polymerase activity.

Discussion

Artichoke tuber tissue contains RNA polymerase activity that is bound to the chromatin and also polymerase activity that is not associated with chromatin under the conditions of preparation, and which appears as a soluble enzyme in the supernatant after chromatin sedimentation. The location of this soluble activity *in vivo* is not known. It is probably of nuclear origin, but not associated with the chromatin, so that the disruption of nuclei which normally occurs during preparation of nuclei or chromatin would release it into the soluble fraction. Certainly RNA polymerase activity with properties very similar to the soluble activity of artichoke and maize (Strain *et al.*, 1971) was isolated from a nuclear-enriched fraction of soya bean (Horgen & Key, 1973), although it is not

clear whether this activity was initially soluble in the nucleus or had been solubilized from the chromatin during the preparation. Polya & Jagendorf (1971a), however, noted that the distribution of soluble and chromatin-bound activity was similar in wheat leaf tissue that had been either homogenized in a Waring Blendor or hand-chopped, and suggested the possibility of a cytoplasmic location for the soluble activity.

The properties of the artichoke soluble polymerases I and II were similar to those of maize with respect to DEAE-cellulose fractionation, sensitivity to salt and response to α -amanitin and rifampicin (Strain *et al.*, 1971). The artichoke activities differed from those of maize in their preference for Mn^{2+} rather than Mg^{2+} , and for denatured rather than native DNA.

The chromatin-bound polymerase activity, but not the soluble activity, was correlated with growth and RNA accumulation in the artichoke tissue. In the presence of 2,4-dichlorophenoxyacetic acid RNA accumulation increased 2.5-fold during 50h and then flattened off, while chromatin-bound and soluble activities increased 2.7- and 1.2-fold respectively over this period and then decreased. Tissues accumulating RNA contained up to 50% of their total polymerase activity bound to the chromatin, whereas those in which there was no RNA accumulation contained only 10–30% chromatin-bound activity. A similar change in the distribution of free and bound RNA polymerase has been noted during the culture of HeLa cells (Ishihama, 1967). Such correlations between growth (RNA accumulation) and the percentage of chromatin-bound polymerase indicate the importance of the association between the polymerase and the chromatin for DNA transcription, and suggest the presence of a pool of polymerase, probably inactive until bound to the chromatin. It should be noted that the increase in chromatin-bound polymerase activity associated with the initial phase of artichoke culture cannot simply be explained in terms of the binding of existing polymerase molecules to chromatin, there being a net increase in total polymerase activity.

The differences between the chromatin-bound and soluble polymerase activities, in terms of response to salt and bivalent cations, do not negate the possibility that the enzyme bound to the chromatin is the same as that in the soluble preparation, since these particular properties could easily be modified by association of the enzyme with the chromatin complex. In order to establish the identity of the chromatin-bound enzyme it is necessary to solubilize it from the chromatin. The polymerase activities solubilized from the artichoke chromatin by a variety of methods were still stimulated by salt, a property which distinguished them from the soluble polymerase activity. However, in these experiments it was not certain whether the polymerase was really free from the chromatin; certainly the solubilized preparation could not be

fractionated on DEAE-cellulose. There is relatively little information available in the literature about the relationship between the enzyme associated with chromatin and that soluble in the cell. From studies on total soluble and total nuclear solubilized activities from wheat leaves, Polya & Jagendorf (1971b) concluded that the activities were very similar. Wheat-germ polymerase activity has been solubilized and characterized by DEAE-cellulose chromatography (Jendrisak & Becker, 1973) and the properties of polymerases I and II are very similar to those of the artichoke soluble polymerases I and II. Unfortunately, however, the solubilized activity was prepared from total wheat germ, in which 80–100% of the total polymerase is soluble (Polya & Jagendorf, 1971a; Mazus & Buchowicz, 1972). The properties of the solubilized polymerase activity should therefore largely reflect those of the soluble activity. The properties of polymerases I and II solubilized from soya bean nuclear material (Horgen & Key, 1973) were basically similar to those of the artichoke soluble polymerases, but again the contributions of soluble activity and solubilized chromatin activity in this nuclear preparation are not clear. Polymerase activity has, however, been solubilized from chromatin isolated from coconut endosperm nuclei, and fractionated by DEAE-cellulose chromatography (Mondal *et al.*, 1972). The fractionation differed somewhat from that of artichoke, maize, wheat and soya bean in that peak I activity was most sensitive to α -amanitin (i.e. analogous to polymerase II) and was also inhibited by low concentrations of rifampicin. Further, both coconut activities were stimulated by about 50% with 0.2M-KCl, a concentration which inhibited artichoke soluble polymerase activity by 70%. In this respect the solubilized artichoke polymerase is similar to the solubilized coconut activity. The available data therefore suggest that the solubilized chromatin polymerase activities may not be identical with those of the soluble preparation, which greatly increases the complexity of any relationship between chromatin-bound and soluble RNA polymerase activities.

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