Activation of Chromatin-Bound DNA-Dependent RNA Polymerase (E.C. 2.7.7.6.) in Plant Storage Tissue Slices

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Chromatin, RNA Polymerase, Potato Tuber Tissue, Aging Phenomenon

The synthesis of RNA by chromatin-bound RNA polymerase (E.C. 2.7.7.6.) from white potato tubers proceeds at a low rate, which is enhanced after slicing the tissue, however. Concomitantly DNA template availability as measured with saturating amounts of *Escherichia coli* polymerase is diminished drastically. Nearest neighbor frequency analysis proved that the RNA synthesized on chromatin of intact tubers is different from that synthesized on chromatin of sliced tissue.

The RNA polymerase of white potato tubers is dependent on all four ribonucleoside triphosphates and a divalent metal ion such as Mg^{2+} or Mn^{2+} and totally inhibited by the presence of pyrophosphate. Actinomycin D blocks the formation of the RNA product, which could be shown to be a heteropolymer by nearest neighbour frequency technique. The K_m of the chromatin-bound enzyme with regard to ATP, GTP, CTP and UTP was 5.1×10^{-5} M, 1.6×10^{-5} M, 0.9×10^{-5} M and 0.45×10^{-5} M/l respectively. α-amanitin inhibits the overall activity to about 50%, which indicates the presence of equal amounts of polymerase I and polymerase II.

The tissue of resting plant storage organs such as white potato tubers can be activated by the simple act of slicing into thin disks and incubation of these fragments in a moist atmosphere for different periods of time ("aging"). Activation is documented by a dedifferentiation of the storage cell to a mitotically active cell on the microscopic level [1] and a de novo synthesis of different messenger RNAs, ribosomal RNAs and transfer RNAs on the molecular level as well [2–5]. Since the synthesis of these macromolecules precedes most of the ensuing metabolic events, the fundamental process of RNA synthesis and its regulation lies at the heart of the "activation phenomenon". Actually experiments on chromatin-directed RNA synthesis in aging storage tissue slices suggest that the induction phenomenon after slicing could be a consequence of altered transcription [6]. The present paper supports this view with experimental evidence for an enhanced RNA polymerase activity on chromatin of aged potato tuber slices which results in the transcription of qualitatively different RNAs inspite of drastically decreased template availability.

Materials and Methods

Plant material

White potato tubers (*Solanum tuberosum* L., cv. "Saskia") of uniform size were stored at 20 °C for at least two days and surface-sterilized for one hour in 0.5% NaOCl before use. Then the tubers were coarsely peeled and the inner tissue was chopped into small squares of about 2 mm^3 volume. These pieces were incubated in 0.25% NaOCl and 5 mM sodium azide for 10 min and thoroughly washed with several changes of sterile water. Either the tissue was used for chromatin isolation immediately or "aged" in moistened, dark desiccators at 25 °C for different periods of time.

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Chromatin isolation

Chromatin was isolated from fresh and "aged" potato tuber tissue according to a modified method of Huang and Bonner (1962) [7]. The tissue was homogenized in 1: 0.6 (w/v) of homogenizing buffer (125 mM sucrose; 100 mM Tris-HCl; pH 8.0; 1 mM $MgCl_2$; 20 mM 2-mercaptoethanol and 5 mM sodium azide together with 0.2 ml of antifoam silicone emulsion M 30 per 100 ml buffer to prevent foaming). After homogenisation in a VirTis "45" homogenizer (30 sec high speed, few seconds low speed, 30 sec high speed) the homogenate was filtered through a double layer of cheesecloth and then through Miracloth. The filtrate was centrifuged at $13000\times g$ for 20 min and the crude chromatin layer was scraped from the underlying starch and cell debris and suspended in 10 mM Tris-HCl, pH 8.0, containing 250 mM sucrose, 10 mM 2-mercaptoethanol and 5 mM sodium azide. The chromatin was recovered by centrifugation, washed three times with the suspension buffer in a Kontes tube and finally layered on top of 20 ml of 1.4 M sucrose in

10 mM Tris-HCl, pH 8.0; 10 mM 2-mercaptoethanol and 5 mM sodium azide. The upper third of the tube was gently stirred and the crude gradient centrifuged in a 3×40 ml swinging bucket rotor Nr. 2417 of the MSE Superspeed 50 at $45000 \times g$ for 2.5 h and at $+4$ °C. The supernatant was discarded and the sucrose-purified chromatin suspended in a small volume of 50 mM Tris-HCl, pH 8.0; 10 mM 2-mercaptoethanol and 5 mM sodium azide. Aliquots of this suspension were used in the assays. All the procedures were carried out with sterile solutions and under sterile conditions.

Standard assay for chromatin-bound RNA polymerase

The assay mixture contained 100 mM Tris-HCl, pH 8.0; 2.5 mM $MgCl_2$; 3.75 mM $MnCl_2$; 2.5 mM dithiothreitol; 2 mM each of GTP, ATP and CTP; 0.033 μmol of [^{3}H]UTP (specific activity 12 Ci/mmol) and 100 μl of purified chromatin corresponding to $5-8$ μg of DNA in a final volume of 0.5 ml. The reaction was initiated by addition of chromatin, routinely carried out for 20 min at 37 °C and stopped by 5 ml of cold 5% trichloracetic acid. The resultant precipitate was transferred to a membrane filter, washed with 2% trichloracetic acid containing 1 mM sodium pyrophosphate and then dried and monitored for radioactivity in a Packard liquid scintillation spectrometer employing a toluene scintillator (containing 100 mg POPOP and 5 g PPO per 1 toluene).

Samples were corrected for radioactivity retained on the filters prepared from unincubated controls. Values for these controls ranged from $5-8$% of the incubated samples.

Template availability

The template availability in the chromatin system was tested using nearly saturating amounts of purified RNA polymerase from *Escherichia coli* MRE 600 in the test mixture.

Nearest neighbour frequencies

For nearest neighbour frequency determination the labelled nucleotide in the assay mixture was scaled up tenfold and the ^{32}P-labelled polynucleotides from this mixture were transferred to glass filter disks (Whatman glass fiber paper, grade 3; 2.3 cm) and washed as described above. The filters were dried and the RNA hydrolysed in 0.5 M KOH for 18 hours at 37 °C. The hydrolysate was neutralized with perchloric acid. The $KClO_4$ and filter disk were removed by centrifugation and washed twice with cold distilled water. The combined extract and wash were lyophilized and then taken up in 0.1 ml of electrophoresis buffer (3.4 ml of pyridine, 34.4 ml of acetic and 3.4 g of sodium ethylendiaminetetraacetate, pH 3.6). Samples were spotted on paper (Schleicher and Schuell Company, Dassel, 2043 a) together with appropriate marker nucleosides or nucleotides. Electrophoresis was carried out in the electrophoresis buffer for 8 h with a potential gradient of 22 V per inch and a current of approximately 18 mA. After electrophoresis, the sheet was dried and cut into individual sample strips, which were further cut into 0.5 cm sections. These pieces were immersed in scintillation fluid and the radioactivity was measured as outlined above.

DNA, RNA and protein determination

Samples were prepared for analysis by precipitating DNA from aliquots of the chromatin preparation in cold 5% ethanolic trichloracetic acid. The precipitates were isolated by centrifugation, suspended in 0.5 N perchloric acid and heated to 70 °C for 1 hour. The DNA content of the hydrolysate was estimated by the diphenylamine colorimetric method of Burton [8]. Highly polymerized salmon sperm DNA was used as standard.

Aliquots of the chromatin solution were diluted appropriately and processed according to the Orcinol method [9] with yeast RNA as chemical for the calibration curve.

Protein content of chromatin samples were determined by a modification of the Lowry procedure [10] with bovine serum albumin as standard.

Results

1. Loss of chromatin-bound RNA polymerase in sucrose density gradients of different molarity

Centrifugation of the crude chromatin material through 1.7 M sucrose gradients constitutes an essential step during chromatin purification according to the Huang-Bonner method. However, as has been shown with soybean hypocotyl chromatin [10, 11] pelleting this material through dense sucrose solutions results in a loss of total RNA polymerase and a selective removal of the α-amanitin-sensitive form. The same holds true for the chromatin-bound enzyme of potato tubers (Fig. 1).

Although the specific activity expressed as pmol UMP incorporated per h and 100 μg DNA rises with rising sucrose concentrations up to 1.4 M, the

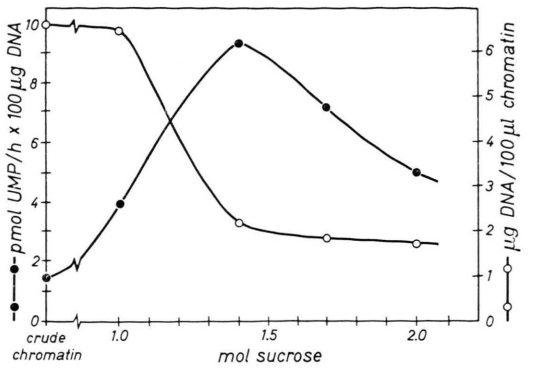


Fig. 1. Recovery of chromatin-bound DNA-dependent RNA polymerase (E.C. 2.7.7.6.) and chromatin DNA of white potato tuber tissue after centrifugation through sucrose gradients of different molarity.

yield of enzyme was appreciably diminished using 1.7 M or 2 M sucrose. So it was decided to pellet the crude chromatin through 1.4 M sucrose solutions.

2. *Slicing-induced alterations in chromatin-bound RNA polymerase activity and template availability*

Chromatin-bound RNA polymerase of sliced tissue is more active than that of resting cells (Fig. 2). The *in vitro* RNA synthesis on both chromatin preparations is complete after about 20 min of incubation. The time course of activation of the enzyme is depicted in Fig. 3 a. Slicing of the tissue induces an activation to about 100% after one day and about 120% after two days of aging. This finding corresponds well with the time course of total RNA synthesis and the synthesis of the different RNA species in this tissue [2]. After two days of incubation the enzyme declines in activity until it reaches the initial level 3 – 4 days after slicing.

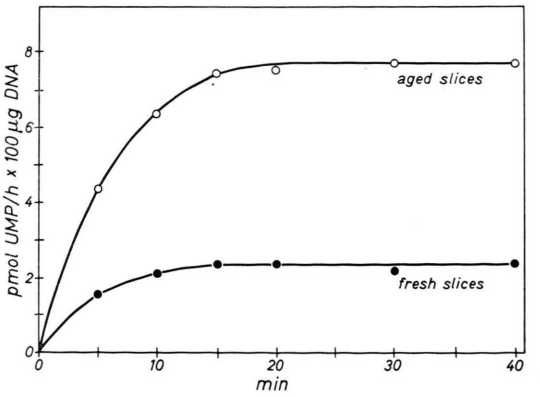
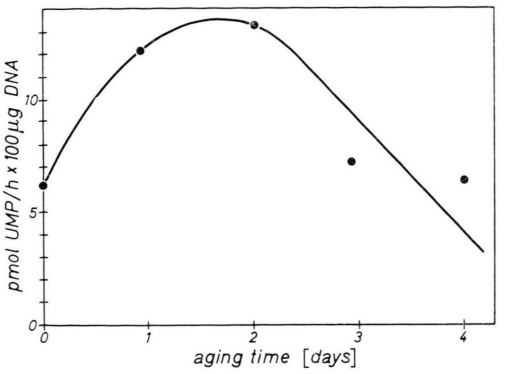
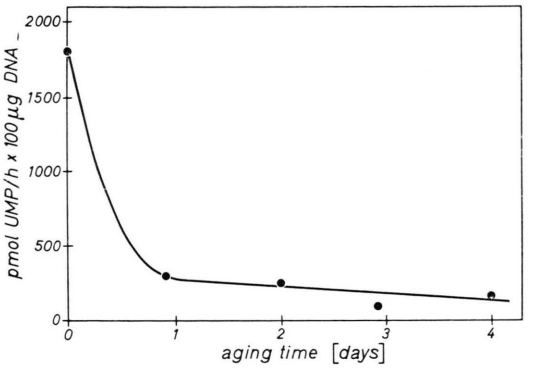


Fig. 2. *In vitro* RNA synthesis by chromatin-bound DNA-dependent RNA polymerase from resting potato tubers ("fresh slices") and aged tissue slices. The reaction mixture contained 100 mM Tris-HCl, pH 8.0; 2.5 mM $MgCl_2$; 3.75 mM $MnCl_2$; 2.5 mM dithiothreitol; 2 mM each of GTP, ATP and CTP; 0.037 mM-UTP and chromatin corresponding to 5–8 µg DNA in a final volume of 0.5 ml.



a



b

Fig. 3. Time-course of activity of chromatin-bound DNA-dependent RNA polymerase (E.C. 2.7.7.6.) after slicing quiescent white potato tuber tissue (a) and slicing-induced alterations in template availability of chromatin (b). Chromatin was isolated and purified from intact tuber tissue and tissue slices after different times of aging and tested for its RNA polymerase activity in the standard assay system (a). Aliquots of the same chromatin preparations were incubated with 30 units of purified *E. coli* MRE 600 RNA polymerase and the incorporation rate was measured in the standard assay mixture (b).

At the same time the DNA template availability as measured with saturating amounts of the RNA polymerase from *E. coli* MRE 600 is diminished drastically (Fig. 3 b). In chromatin of resting cells the incorporation rate supported by the *E. coli* enzyme is about 300 times that of the endogenous

enzyme. After slicing the availability drops to about 25% of the initial after 24 hours and levels off afterwards. Thus the endogenous enzyme enhances its activity after slicing in spite of the fact that overall template availability is diminished.

3. *Characterization of chromatin-bound RNA polymerase of potato tubers*

Up to a concentration of 5 μg of chromatin-DNA the reaction rate of the enzyme is directly proportional to the amount of DNA in the reaction mixture. At about 15 – 20 μg DNA the enzyme is saturated (Fig. 4). Lineweaver-Burk plots give an apparent K_m of 3.33 μg DNA/0.5 ml of reaction mixture.

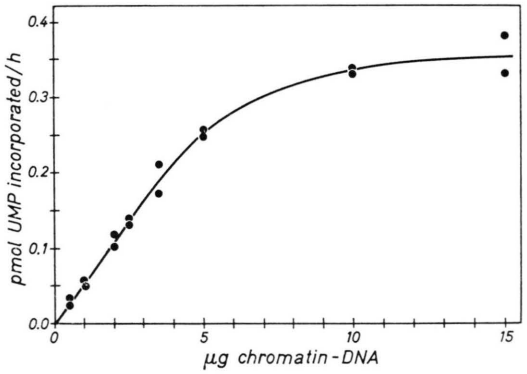


Fig. 4. *In vitro* RNA synthesis by potato RNA polymerase as a function of chromatin-DNA concentration.

Total saturation of the chromatin-DNA with *E. coli* polymerase could not be achieved using even more than 30 units of the enzyme, one unit corresponding to an incorporation of 1 nmol AMP into RNA material per 10 min [12]. Though the incorporation proceeds at a linear rate even at 30 units of RNA polymerase, saturation experiments were carried out with that activity in the test mixture (Fig. 5).

The properties of the white potato polymerase system are mostly identical with those of other systems [6]. Enzyme activity was totally dependent on the presence of a divalent cation such as Mg^{2+} or Mn^{2+}, in this case Mn^{2+} being the more effective ones. Omission of the nucleotides results in reduction of the reaction rate, but never approached lower values than 50% of the complete test system. Possibly the purification procedure did not remove all endogenous nucleotides from the chromatin.

Table I. Properties of chromatin-bound RNA polymerase of white potato tuber tissue. The values are the average of replicate assays and expressed as a percentage of the control assay. (100% = 1.698 pmol UMP/h per 100 μg DNA).

System		Percentage of control
Complete		100
— NTP		30.8
— Mg^{2+}		99.2
— Mn^{2+}		30.6
— Mg^{2+}/Mn^{2+}		1.1
— DTT		91.0
Phosphate	12.5 mmol	115.0
	25.0 mmol	105.3
	50.0 mmol	101.4
Pyrophosphate	12.5 mmol	4.6
	25.0 mmol	1.5
	50.0 mmol	0.7
RNAse	0.5 μg	7.5
	5.0 μg	6.2
	50.0 μg	6.6
DNAse, added 30 min before start (50 μg)		24.8
DNAse during reaction (50 μ)		21.8
Trypsin, added 30 min before start (50 μg)		42.5
Trypsin uring reaction (50 μg)		42.5
100 °C (10 min)		13.0

Pyrophosphate totally inhibits the enzyme, phosphate proved to be ineffective. The reaction is RNAse- and DNAse-sensitive and proceeds at a slower rate, if trypsin is present (Table I). Further, the amount of product accumulated is minimized if actinomycin D is added to the reaction mixture. Less than 5 μg of the drug per ml inhibits the activity to about 70%.

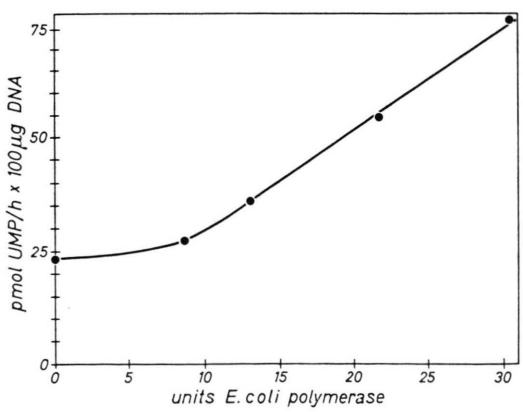


Fig. 5. Saturation of white potato tuber chromatin with increasing concentrations of *E. coli* MRE 600 RNA polymerase.

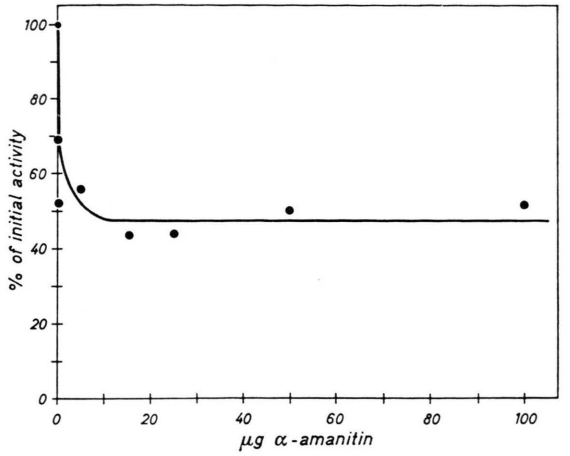


Fig. 6. Discrimination between RNA polymerase I and RNA polymerase II of white potato tuber chromatin by the presence of α-amanitin in the *in vitro* assay system.

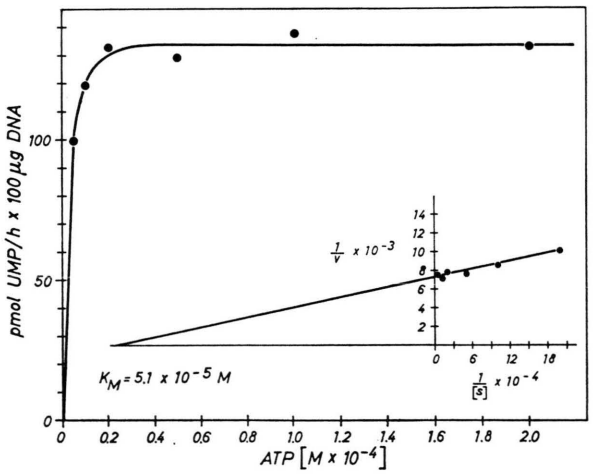


Fig. 7 a.

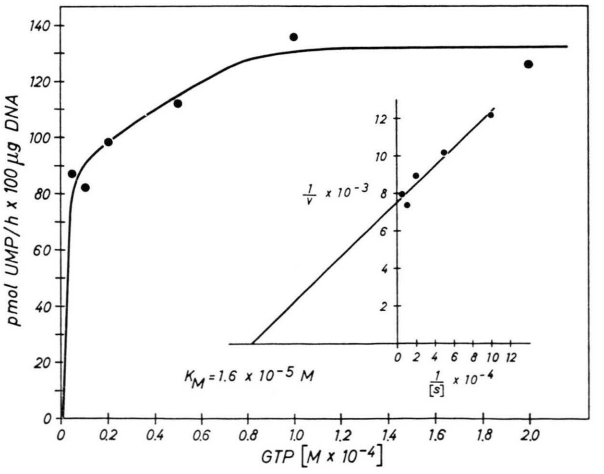


Fig. 7 b.

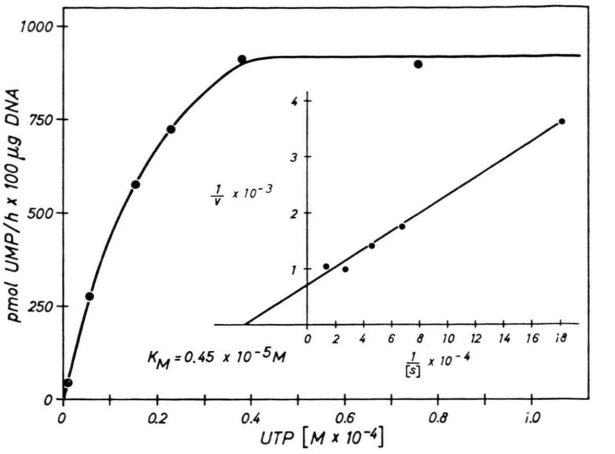


Fig. 7 c.

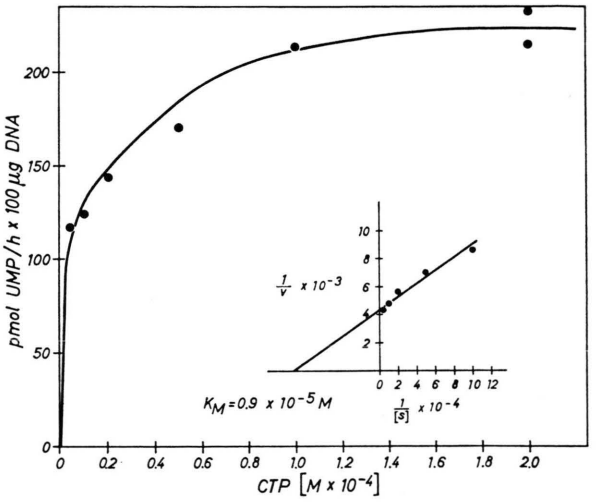


Fig. 7 d.

Fig. 7. Dependence of the polymerization rate on the concentrations of the four nucleotides ATP (a), GTP (b), UTP (c) and CTP (d). The reaction mixture contained in a final volume of 0.5 ml: 100 mM Tris-HCl, pH 8.0; 2.5 mM $MgCl_2$; 3.75 mM $MnCl_2$; 2.5 mM dithiothreitol; 2 mM of each nucleotide with the nucleotide tested in the concentrations indicated. The reaction mixture was incubated for 5 min at 35 °C. The insets represent Lineweaver-Burk plots of the corresponding data.

The α-amanitin-sensitive proportion of the total polymerase system, presumed to be polymerase II, amounts to about 50% as judged from inhibitor studies, At a concentration of less than 5 μg/ml the inhibition of the enzyme II is maximal (Fig. 6).

All the four nucleotides tested act as substrates in the polymerization reaction. The rate of polynucleotide synthesis as a function of individual nucleotide concentrations is depicted in Fig. 7. Plotting the

data on double reciprocal axes according to Lineweaver-Burk, which is presented as inserts, yields an apparent K_m-value fo 5.1×10^{-5} M for ATP, 1.6×10^{-5} M for GTP, 0.45×10^{-5} M for UTP and 0.9×10^{-5} M for CTP respectively.

4. Characterization of the RNA synthesized on isolated chromatin of resting and activated cells

The nearest neighbor frequency of the RNA product of chromatin-bound RNA polymerase from resting and activated cells was determined using [α-^{32}P]ATP. The product was hydrolyzed with KOH and the individual nucleotides separated by paper electrophoresis (Table II). It is apparent that the RNA synthesized on chromatin of resting cells is different from that of activated ones. The most important tendency in all experiments is an increase in A – A-linkages with concomitant decrease in C – A-bonds of the RNA produced by the endogenous enzyme. The nearest neighbor frequency of the RNA synthesized by *E. coli* polymerase on the template of resting cells, however, shows no significant differences from that produced on template of mitotically active cells (Table II).

Table II. Nearest neighbor analysis of RNA produced by chromatin-bound RNA polymerase and by chromatin saturated with *E. coli* RNA polymerase. Chromatin from fresh and "aged" potato tuber tissue was isolated, purified and incubated together with 4.762 nM of α-[^{32}P]ATP with and without 30 units of *E. coli* polymerase for one hour. The ^{32}P-labeled polynucleotides were then processed as described in Materials and Methods. The values are the average of four independent experiments.

		Distribution of label in each 2′,3′-mononucleotide [mol %, s. d. ±1.2]			
Aging time	[h]	CMP	AMP	GMP	UMP
Endogenous polymerase	0	21.4	26.5	27.0	25.2
	24	18.4	29.6	28.1	23.9
E. coli polymerase	0	17.5	30.6	28.5	23.5
	24	18.7	29.4	28.3	23.6

Discussion

The rapid, slicing-induced polyribosome formation in potato tuber tissue is dependent on previous transcription [2], which in turn is activated immediately after slicing. An indication for this activation process is the time-dependent rise in chromatin-bound RNA polymerase activity during the first 24 – 48 hours. Aging for an additional 24 hours causes a drastic decline of this activity to the level of resting tissue. This is true for most other enzymes of sliced tissues and metabolic activities like respiration and protein synthesis generally [1, 4].

The time-course of chromatin-associated RNA polymerase also roughly parallels the RNA synthesizing capacity of the aging slices. The RNA synthesized by the endogenous RNA polymerase after 24 hours of aging is different from that synthesized by the enzyme from resting tissue. Nearest neighbour analysis shows an increased proportion of A – A-linkages after slicing, which may be taken as indication for A-rich sequences in the RNA and hence for mRNA. At the same time the number of A – C-neighbours decreased. So it can be concluded that another set of genes is transcribed as a consequence of slicing the tissue. The nearest neighbour frequency of the RNAs produced by exogenously added *E. coli* polymerase on chromatin preparations from intact tubers and aged slices, however, proved to be very similar, inspite of the fact that chromatin of aged tissue slices has a greatly reduced template availability (Table II). One possible explanation for the drastic decline in available genes with no appreciable alteration of the quality of the gene transcripts points to a reduction of freely accessible redundant genes in aging tissue. On the other hand it is known that the initiation sites on *i. e.* mammalian chromatin recognized by *E. coli* RNA polymerase in at least some cases are different both qualitatively and quantitatively from those occupied by the endogenous enzyme [13, 14]. So the experiments reported here at best show that the chromatin does undergo changes after slicing. These changes prevent heterologous RNA polymerase from approaching new initiation sites, whereas the homologous enzyme in contrast evidently recognize a larger number of these sites on the template.

Actually more DNA template sites must be free for transcription than can be transcribed by the endogenous polymerase. Saturation of chromatin with *E. coli* polymerase at any time enhances the rate of RNA synthesis appreciably. So, with chromatin from intact tubers the stimulation was about 300 times and with chromatin from aged slices still some 25 times that of control tissue. But, notwithstanding the increase in RNA polymerase activity after slicing, the template availability decreases drastically at the same period. This is in contradiction with

the results obtained in sugar beet experiments [6] and as yet not explainable. However, the template available varies considerably with the developmental stage of the tubers as does the endogenous RNA polymerase activity. Evidently high mitotic activity coincides with increased polymerase action and decreased availability of DNA to *E. coli* polymerase (unpublished results).

The chromatin-bound RNA polymerase of potato tuber tissue possesses similar properties as the enzymes from various other plant tissues [6, 5–21]. It requires the presence of a divalent metal ion and all four nucleoside triphosphates for activity. The synthesis of the heteropolymeric product is inhibited by pyrophosphate and partly by actinomycin D and α-amanitin. Since α-amanitin in the concentrations used exclusively inhibits polymerase II [22, 23], it can be concluded from a 50% inhibition of the overall activity that polymerase I and polymerase II are present in equal activities (amounts?) on the chromatin of resting tissue.

Although several hypotheses have been put forward in order to explain the activation phenomenon in sliced plant storage tissue [1], none of them has been proved experimentally. However, it is certain that the control of genetic activity – in other words the regulation of the DNA-dependent RNA polymerase in these tissues – is a crucial point in this process.

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