Transcription and Release of RNA in Isolated Nuclei from Parsley Cells

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An *in vitro* transcription system using nuclei from freely suspended callus cells of *Petroselinum crispum* is described. The use of a filtration technique allowed the measurement of transcription and release of RNA simultaneously over very short time intervals. The transcription showed a biphasic time course, with an early maximum at 2 and a later one at 20 minutes. The early maximum was ascribed to the activity of polymerase II (α-amanitin sensitive), the later to that of polymerase I. While the transcriptional process was independent of the temperature used for incubation up to 26 °C and even increased with temperatures above that, the release of RNA transcribed was inhibited by temperatures above 36 °C.

Introduction

Eucaryotic cells, compared with procaryotes, have a very different organisation with additional possibilities for regulating their genome-dependent cellular metabolism. These include among others: 1. Multiple DNA-dependent RNA-polymerases with specific properties, 2. a strict compartmentation between nucleus and cytoplasm with the consequence of a spatial separation of transcription and translation, 3. regulation at the level of precursor molecules for rRNA and mRNA and 4. regulation of the transport of ribonucleoprotein particles from nucleus to cytoplasm. For the investigation of these processes *in vitro* systems such as isolated nuclei are required.

In this communication an isolation procedure for nuclei from parsley cells grown in suspension culture is described. The incorporation of [^{3}H]UMP into TCA insoluble molecules was measured by a special filtration technique which allowed the determination of the transcription and the release of RNA simultaneously over very short intervals. Data are presented here which support the idea that two RNA polymerases are active in our isolated nuclei. Results from experiments dealing with the temperature dependence of the transcription process support our hypothesis, that transcription and release are two independent processes.

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Materials and Methods

1. Plant material

Cells of *Petroselinum crispum* were propagated as previously described [1]. Cells from the logarithmic stage (5 days after inoculation) were used for the preparation of nuclei.

2. Solutions

Isolation medium 1 (IM 1) contained 20 mM Tris·HCl (pH 7.8), 200 mM sucrose, 2 mM $CaCl_2$, 10 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 10 mM KCl, 2.5% Ficoll (w/v) and 5% Dextran T40 (w/v). Isolation medium 2 (IM 2) was like IM 1 but containing in addition 0.02% Triton X-100. Isolation medium 3 (IM 3) equalled IM 1 without sucrose.

3. Preparation and purification of nuclei

Nuclei were isolated by a modification of the methods of Tautvydas [2] and Gebauer *et al.* [1] and purified by a modified method of Chen *et al.* [3]. Cells (fresh weight 50 g) were incubated in IM 1 containing 2% pectinase (Serva, Heidelberg) and 1.5% cellulase (Onozuka R10, Yakult Biochemicals, Japan) at 30 °C for 4 h. The resulting crude protoplasts were collected on a 100 μm nylon net and brought to a final volume of 160 ml with IM 1. The suspension was homogenised in a Potter-Elvehjem homogeniser (glass/teflon). After filtration through nylon nets with decreasing pore sizes of 200, 140, 100, 45, 30 and 10 μm the suspension

was centrifuged at $1200 \times g$ for 10 min in a Sorvall centrifuge using the HB-4 rotor. The pellet was resuspended in IM 2 and sedimented once more at $1200 \times g$ for 10 min to remove the cytoplasmic material adhering to the nuclear envelope [4, 5].

The nuclear pellet was resuspended in IM 1, layered on a sucrose cushion (23 ml IM 3 containing 1.2 M sucrose) and centrifuged at 20 000 rpm for 7 min at 2 °C in a Beckman centrifuge using a SW 25.1 rotor. The nuclear pellet was now resuspended in IM 3 containing 1.0 M sucrose, centrifuged through a discontinuous gradient consiting of 10 ml each of 1.2, 1.4, 1.8 M sucrose for 10 min at 4000 rpm using a SW 25.1 rotor. The purified nuclei were collected from the 1.2 and 1.4 M zones. The nuclei could be stored at − 20 °C for 8 days without losing their activity if 40% (v/v) glycerol was present. A 10 min centrifugation at $1200 \times g$ freed them from the storage medium. For measurement of transcription the nuclei were suspended in IM 1 at a concentration of 8×10^6 nuclei/ml.

4. RNA synthesis in isolated nuclei

The complete incubation medium modified from Hamilton *et al.* [6] and Blaschek *et al.* [7], kept in ice, contained 20 mM Tris · HCl (pH 7.8), 200 mM sucrose, 10 mM 2-mercaptoethanol, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 10 mM KCl, 1 mM $CaCl_2$, 1.25% Ficoll, 2.5% Dextran T40, 1 mM each of ATP, GTP, CTP, UTP, 0.3 μCi [5-^{3}H]uridine 5′-triphosphate (Amersham Buchler, Braunschweig) and 0.25 ml of nuclei suspension in IM 1 in a total volume of 0.5 ml, added last in order to start the transcription reaction. Yeast RNA (10 μg/ml) was also present. The temperature of the incubation was 36 °C unless mentioned otherwise. Transcription was stopped by adding 0.1 mg bovine serum albumin (Calbiochem) and 0.1 M sodium pyrophosphate ($Na_4P_2O_7$) dissolved in 0.5 ml of double distilled water. The nuclei (I) were collected on Millipore filters (RA 1.2 μm, ϕ 25 mm) by a careful filtration step.

The filtrate (II) resulting from this step contained the incubation mixture and the solution used to terminate the reaction (see above).

The filter discs bearing the nuclei (I) were treated with 20% TCA (in 0.01 M $Na_4P_2O_7$). The filtrate (II) was treated immediately with 1 ml of 20% TCA dissolved in 0.01 M $Na_4P_2O_7$ and subsequently with 30% TCA (in 0.01 M $Na_4P_2O_7$). In both cases (I, II), the filters bearing the TCA insoluble material were washed two times with 20% TCA and once with 96% ethanol at − 20 °C. The dry filter discs were counted for radioactivity in toluene-PPO-POPOP.

5. [^{3}H]UTP uptake by isolated nuclei

Nuclei were sedimented at $50 \times g$ for 5 min, washed two times with IM 1 ($100 \times g$, 5 min) and suspended in 0.5 ml 2% SDS for lysis [9]. For total uptake measurements 0.1 ml of the lysate was transferred into Aquasol (NEN Chemicals, USA) and counted for radioactivity. The remaining lysate was treated with 20% TCA containing 0.01 M $Na_4P_2O_7$. The precipitate was collected on glass fiber filters, washed, dried and counted to measure the incorporation of [^{3}H]UMP into acid precipitable material.

6. Determination of nuclei concentration

For determining the nuclei concentration the suspension was mixed with an equal volume of acetoorcein (1 g orcein in 100 ml of 70% acetic acid) and nuclei were counted in a haematocytometer.

7. Cell number determinations

Cells were counted according to Grierson and Covey [10]. Filtered cells were suspended in 15% chromic acid (1 ml chromic acid per 0.2 g fresh weight) and incubated at 60 °C for 20 min. The cell clusters were homogenised by siphoning them backwards and forwards in a Pasteur pipette. The reaction was stopped by adding 4 ml NaCl-formaldehyde (5 μl formaldehyde per l 0.5% NaCl) and the individual cells were counted in a haematocytometer.

Results

In vitro transcription

Nuclei, prepared from cells of a suspension culture of Petroselinum crispum were obtained in following yields (as compared with the primary cell number): 60 – 80% before, 15 – 30% after the final purification step. The RNA synthesis was measured by the incorporation of [^{3}H]UMP into the TCA precipitable fractions. By means of the filtration technique used in this investigation it was possible to determine the RNA transcribed by the isolated nuclei and the RNA released from these nuclei simultane-

ously. It should be also emphasized that with the method described here we were able to determine the incorporation very early after the reaction had been started by adding the cold nuclei suspension to the incubation mixture. The kinetics of the incorporation reaction showed two peaks in the activity (Fig. 1), the first one being evident within two minutes, the second one having the appearance of a plateau at 20 min. The incubation mixture contained an excess of yeast RNA. If this component was omitted the biphasic behavior was maintained, but the curve declined after 20 min (data not shown), indicating that the yeast RNA was able to reduce degradation of the newly synthesised RNA. The second phase of the UMP-incorporation was a saturation curve, typical for this type of experiment. The very fast reaction in the beginning necessitated to start with cooled nuclei suspensions (4 °C). With a centrifugation method [9] the amount of free UTP in these nuclei was determined (Table 1). The amount of radioactivity taken up by the nuclei was nearly constant over the time of incubation. The insoluble material, recovered from the lysate by centrifugation showed the same biphasic behavior, but with lower incorporation values compared to Fig. 1. The time course of the changing UTP-pool showed reciprocal values compared with the time course of the incorporation into the TCA insoluble material indicating a true incorporation of UMP into polyribonucleotides. The average value for the UTP-pool determined in this experiment was 0.65 nmol $\times 10^{-6}$ nuclei. In comparison to nuclear preparations from other material [7] parsley nuclei had a very high incorporation rate (45 pmol $\times$ 10^{-6} nuclei $\times$ min^{-1}).

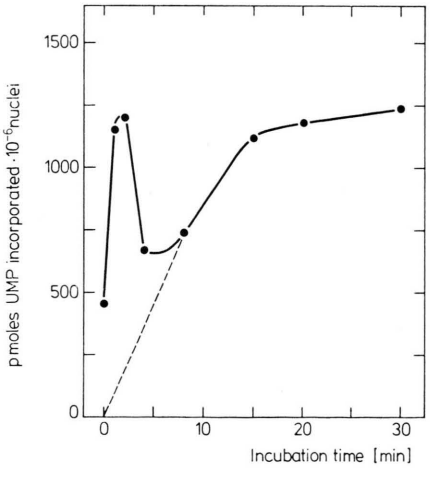

Fig. 1. Kinetics of [^{3}H]UMP incorporation into RNA by isolated nuclei from *Petroselinum crispum*. Isolation of nuclei and conditions for incubation are described in Materials and Methods (chapter 4). The nuclear suspension was added to the incubation mixture to start the reaction. Temperature was 36 °C. The radioactivity of the total TCA insoluble fraction is shown. Blank values have not been subtracted. The curve represents mean values from 2 parallel samples. The same time course was obtained in all experiments (5) carried out.

Table I. Determination of [^{3}H]UTP uptake and incorporation of [^{3}H]UMP into TCA insoluble material according to [9]. After the incubation the nuclei were sedimented at 700 rpm for 5 min, washed 2 times by centrifugation (1400 rpm, 5 min) with IM 1 and suspended in 0.5 ml 2% SDS. An aliquot (100 μl) of the lysate was counted for radioactivity (total uptake). To the remaining lysate 0.5 ml of 20% TCA containing 10 mM sodium pyrophosphate were added. The precipitate was collected on glass fiber filters and counted for radioactivity (TCA insoluble material). (For special conditions see Materials and Methods, chapter 5.)

	pmol UTP uptake and incorporation by 10^6 nuclei		
Time of incubation [min]	Total uptake (uptake and incorporation)	TCA insoluble material	TCA soluble material *
0	1159	40	1119
2	961	755	206
4	1265	444	820
20	1460	1013	447

* Calculated from total uptake and TCA insoluble material.

α-amanitin experiments

In order to get some information about the two processes apparently occuring in the nuclei from parsley, experiments with α-amanitin were carried out, since we suspected, that the biphasic behavior was the result of two transcription processes driven by different RNA ploymerases. In Fig. 2 the results from these experiments are presented. After the addition of 5 μg/ml α-amanitin the peak at 2 min was prevented and the slower plateau-type process was reduced to 62% of the control values. Thus it seemed that the early peak in the transcription process should be ascribed to the activity of polymerase II, normally sensitive to α-amanitin and responsible for the synthesis of hnRNA, whereas the second phase would seem mainly the result of the activity of polymerase I, responsible for the rRNA synthesis and normally insensitive to α-amanitin. The reduction to 62% can possibly be explained by inhibition of polymerase III [11].

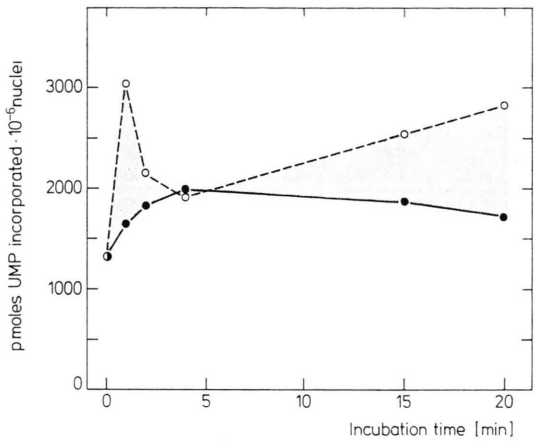

Fig. 2. Kinetics of [^{3}H]UMP incorporation into RNA by isolated nuclei. Treatment with α-amanitin (5 μg/ml). Incubation conditions and radioactivity determinations were the same as in Fig. 1. ○– – –○ control, ●———● α-amanitin treated nuclei.

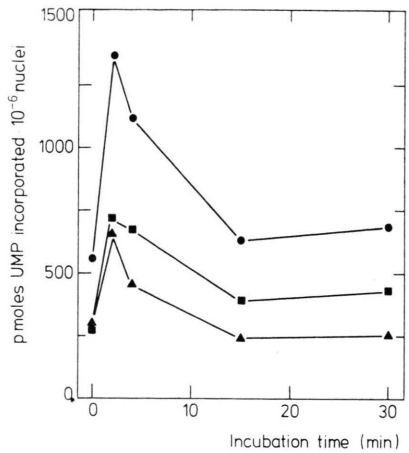

Fig. 3. Kinetics of [^{3}H]UMP incorporation into RNA by isolated nuclei stored for 20 days at −20 °C in 40% glycerol. For incubation conditions and radioactivity determinations see Fig. 1. ■———■ incorporation of [^{3}H]UMP into RNA of the nuclear fraction (I; RNA transcribed but not released). ▲———▲ TCA insoluble material in the filtrate, separated by filtration from the nuclear fraction (II; see Materials and Methods), representing the RNA transcribed and released from the nuclei. ●———● the total [^{3}H]UMP incorporated, calculated by addition of the two other curves (I+II).

Both processes are sensitive towards actinomycin D (18 μg/ml), thus indicating transcription in our system. The inhibition was 74% at the 2 min peak and 60% at 20 min.

In nuclei stored at −20 °C in 40% glycerol the capacity to transcribe RNA was maintained for 8 days. After a storage period of 20 days the slower process did not take place, whereas the α-amanitin sensitive process with its peak at two minutes was maintained (Fig. 3). This fact gives us a further hint that the biphasic time course is the result of the activity of at least two different RNA polymerases present in these isolated nuclei from parsley.

Temperature dependence of transcription and release of RNA

After 15 minutes of incubation at different temperatures [^{3}H]UMP incorporation into nuclei was determined. In Fig. 4 it can be seen that the rate of incorporation was fairly constant over a temperature range of about 8 °C – 26 °C. At temperatures exceeding 26 °C transcription in the nuclei increased, while in the filtrate representing the RNA transferred from nuclei into the medium, the radioactivity decreased. These results demonstrate that synthesis and release of RNA were two processes, measurable independently. Furthermore it could be shown that the nuclei did not loose their newly synthesised RNA at temperatures above 36 °C as isolated nuclei do from animal cells (Ventling, personal communication).

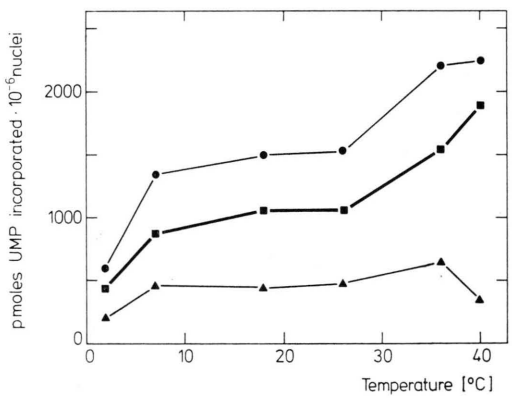

Fig. 4. Temperature dependence of the [^{3}H]UMP incorporation into RNA after 15 min of incubation. ■———■ incorporation of [^{3}H]UMP into RNA of the nuclear fraction (I; RNA transcribed but not released). ▲———▲ TCA insoluble material in the filtrate (II), separated by filtration from the nuclear fraction (see Materials and Methods, chapter 4), representing the RNA transcribed and released from the nuclei. ●———● the total [^{3}H]UMP incorporated, calculated by addition of the other curves (I+II).

Discussion

In the present study an isolation procedure for nuclei from freely suspended callus cells of parsley is described. Furthermore the use of these nuclei for studying transcription *in vitro* was also investigated.

The determination of the radioactivity (incorporated into the TCA-insobluble material) by a filtration technique enabled us to examine both transcription and the release of newly synthesised RNA simultaneously. In our *in vitro* system the nuclei synthesised RNA up to at least 20 min. The transcription in nuclei from these plant cells was a biphasic process with a maximum at 2 and 20 min. Addition of α-amanitin, another sensitive enzyme such as polytion. This result lead to the conclusion that the early reaction was due to the activity of polymerase II. The slower plateau-type process was less affected by the drug. An explanation might be that in addition to polymerase I, normally insensitive towards α-amanitin, another sensitive enzyme such as polymerase III was active at that time. In nuclei from animal cells very similar results could be obtained *in vivo* [12].

The degradation of newly synthesised RNA in the nuclei could be reduced by the addition of yeast RNA. Assuming that the RNase activity is constant over the whole incubation period, the biphasic behavior of the curve could only be ascribed to at least two transcriptional activities. Furthermore the sensitivity towards α-amanitin (see above) strongly supports this interpretation.

Due to the very fast reaction in the beginning it was necessary to start with a cold nuclear suspension to detect the early maximum at 2 min. With suspensions preincubated at 36 °C the activity of the α-amanitin sensitive polymerase could not be detected.

The activity of at least two polymerases was also evident from the stability of the enzymes during storage. After 20 days the activity of the slower process but not of the rapid one was lost. Polymerase II, apparently, was still active and was much more stable. Differencies of stabilities for these two polymerases from parsley were also reported by Link and Richter [13]. Future experiments will prove the specifity of the RNA transcripts of the two RNA polymerases and also shed some light on the mechanism of RNA release.

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