Transcription in nuclei isolated from carrot protoplasts: effects of exogenous DNA

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1. INTRODUCTION

Various types of isolated plant nuclei synthesize RNA in vitro [1–8]. Exogenously supplied DNA could be transcribed and RNA stimulated synthesis in isolated plant nuclei [7,8]. This suggests that foreign DNA taken up by, e.g., plant protoplasts might be transcribed in vivo. Furthermore, liposome-mediated DNA uptake by carrot protoplasts has been demonstrated [9,13], part of the donor DNA being found associated with recipient nuclei. Thus, our goal is to develop a simple plant in vitro transcription system allowing us to determine whether plant RNA polymerase II is able to achieve meaningful transcription of cloned prokaryotic and eukaryotic genes.

In vitro RNA synthesis in carrot nuclei has not yet been described. Since our interest is to genetically engineer carrot protoplasts, we have determined conditions under which isolated carrot nuclei actively synthesized RNA. We also show that exogenous DNA stimulated in vitro RNA synthesis as observed in [8]. Such stimulation could be due in part to the inhibition of ribonuclease present in the nuclei preparations.

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2. MATERIALS AND METHODS

Ribonuclease free sucrose and sodium dodecylsulfate were purchased from BioRad and Percoll from Pharmacia. [^{3}H]UTP (44 Ci/mmol) was from ICN. All other biochemicals were from Sigma. Glassware was washed in a solution of 0.8% (v/v) diethylpyrocarbonate and oven-baked.

2.1. *Isolation of nuclei*

Carrot (*Daucus carota* L.) cells were grown in suspension as in [12]. The packed cell volume (PCV) doubled in ~ 60 h. Protoplasts were generated by incubating cells for 15 min in 0.4 M mannitol buffered with 0.05 M potassium citrate (pH 4.8) containing 1% (w/v) cellulase R-10 (Yakult Biochemicals) and 0.1% (w/v) Pectolyase (Sieshiu Pharmaceuticals). Protoplasts were washed 4 times in buffered mannitol containing 1 mM EGTA and 0.1 mM spermine–HCl. They were then lysed at 0°C for 5 min with 0.1% (v/v) Triton X-100 dissolved in 0.15 M sucrose containing 5 mM $MgCl_2$, 1 mM DTT, 5 mM MES buffer (pH 6.1), 1 mM EGTA and 0.1 mM spermine–HCl. All subsequent steps were performed at 4°C. The lysate was spun at 600 × *g* for 5 min after under-layering with 0.25 M sucrose containing 5 mM $MgCl_2$, 1 mM DTT, 5 mM Hepes (pH 7.9), 1 mM EGTA and 0.1 mM spermine–HCl. The greyish-white pellet was then resuspended in 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM DTT and 5 mM Hepes (pH 7.9). This suspension was placed on top of a 13.5 ml 100%, 80%, 60% (v/v) Percoll step gradient [2,3,6]. The 80% and 60% Percoll contained sucrose, $MgCl_2$, DTT and Hepes as above. All layers were adjusted to pH 7.9 with 1 M NaOH. The

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gradients were centrifuged in a Beckman SW 40 rotor at 4°C for 15 min at 4000 rev./min. The nuclei that banded at the 80%/60% interface were collected and rinsed twice with 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM DTT and 5 mM Hepes (pH 7.9). The final pellet was resuspended in 50% (v/v) glycerol, 2.5 mM $MgCl_2$, 0.5 mM DTT, 2.5 mM Hepes (pH 7.9) and stored frozen at – 70°C.

Nuclei counts were done using a Spencer hemacytometer. Nuclear DNA content was determined using the modified diphenylamine assay in [14]. Microscopically, these nuclei appeared ellipsoidal and stained readily with toluidine blue 0. They had a clearly visible nucleolus and were free from starch grains or other cytoplasmic contaminants. Nuclei yields based on a count of protoplasts ranged from 23–54%. Each nucleus contained 4.1 pg DNA.

2.2. *Assay for in vitro RNA synthesis*

The optimum transcription mixture contained up to 500 000 nuclei in 50 μl polymerase cocktail containing final concentrations of 10% (v/v) glycerol, 150 mM $(NH_4)_2SO_4$, 70 mM KCl 15 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, 10 mM Hepes (pH 7.9), 0.1 mM EDTA, 0.1% (w/v) BSA, 1.6 mM phosphoenol pyruvate, 1.6 mM spermine–HCl, 0.2 mM ATP, 0.2 mM GTP, 0.2 mM CTP, 0.02 mM UTP, 2.5 μg pyruvate kinase and 20 μg salmon sperm DNA. [^{3}H]UTP was added to 20 μCi/ml of cocktail. The reaction mixture was generally incubated at 29°C for 30–40 min. Changes in these conditions are detailed in the figure legends. The reaction was terminated by adding sarkosyl to 1% (w/v) final conc. and disodium EDTA to 20 mM and placing on ice. Trichloracetic acid-insoluble radioactivity was collected on GF/F filters (Whatman), rinsed with cold 95% ethanol, dried and counted in Fisher scintillation counting fluid. The scintillation counter used was a Beckman LS 7000. In vitro synthesized RNA was extracted as in [6].

2.3. *Sedimentation coefficient determinations*

Sucrose gradients of 5–20% (w/v) containing 1.0 M NaCl, 0.01 M EDTA, 0.1% (w/v) sarkosyl and 0.1 M Tris–HCl (pH 7.5) at 22°C were prepared in 5 ml polyallomer centrifuge tubes. Centrifugation was for 3 h at 40 000 rev./min using an SW 50.1 rotor (Beckman). All samples of extracted RNA were heated to 100°C for 2 min and then quickly cooled before placing onto gradients. Gradients were pumped from the bottom of the tube using a Beckman fractionator. Fractions were collected and trichloroacetic acid insoluble radioactivity was determined as above.

S-Value determinations were made by comparing the sedimentation patterns to a 28 S rRNA marker from bovine testes. Calculations were made based on the empirical formulae in [15].

2.4. *Assay for endogenous nuclease activity*

Bovine testicular 28 S r[^{14}C]RNA (kindly provided by W.P. Cheevers) was incubated with nuclei, with or without double-stranded salmon sperm DNA. These samples were then made to 1% (w/v) sarkosyl, heated to 100°C, chilled and sedimented through neutral sucrose gradients as above.

2.5. *Determination of polyadenylated RNA*

RNA extracted as above was loaded onto and eluted from 0.4 g oligo(dT)-cellulose columns as in [16]. Counting aliquots from samples was as above.

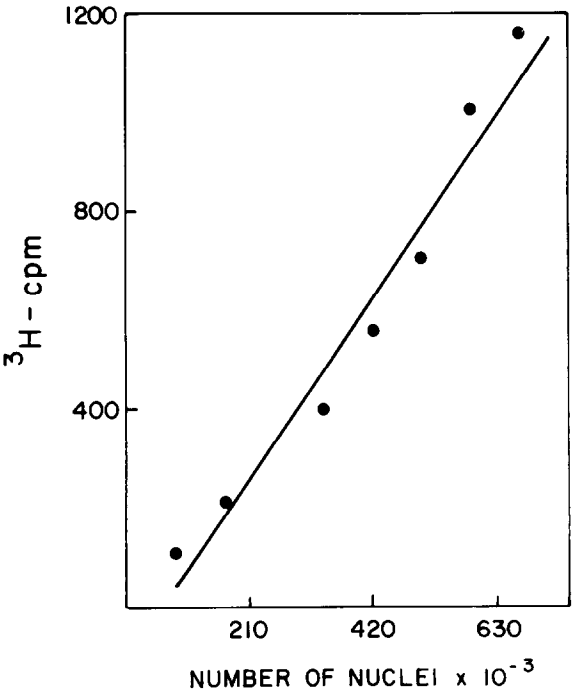

Fig.1. Concentration dependence of nuclei on in vitro RNA synthesis in carrot nuclei. Varying numbers of nuclei were assayed for amounts of trichloroacetic acid-insoluble radioactivity after 30 min incubation at 29°C. Results are the average of duplicate samples.

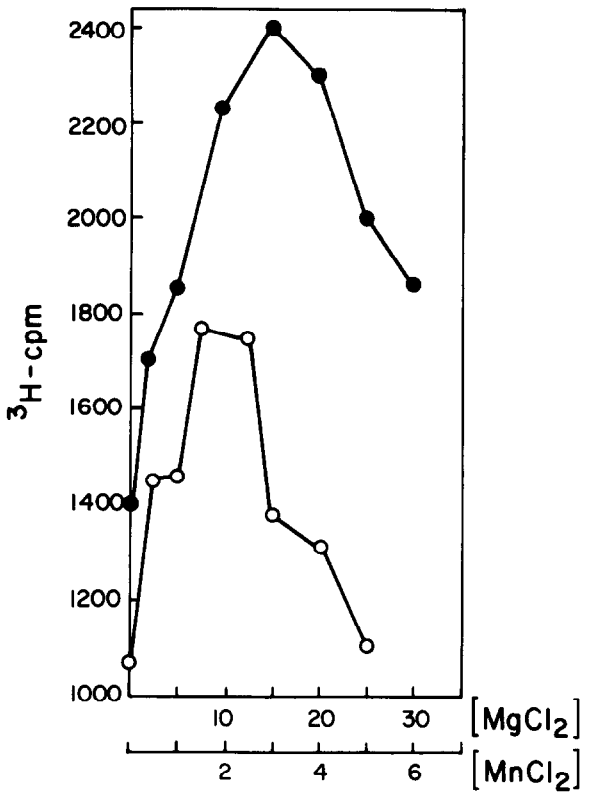

Fig.2. Effects of divalent cation concentration on RNA synthesis activity in carrot nuclei. Nuclei containing zero or the highest salt concentration indicated were mixed to yield the final concentrations indicated. All samples contained 150 mM $(NH_4)_2SO_4$. The Mn^{2+} samples (○—○) each contained 2.5 × 10^5 nuclei. The Mg^{2+} samples (●—●) contained 4.2 × 10^5 nuclei each. Incubation was at 29°C for 40 min. Results are the average of duplicate samples.

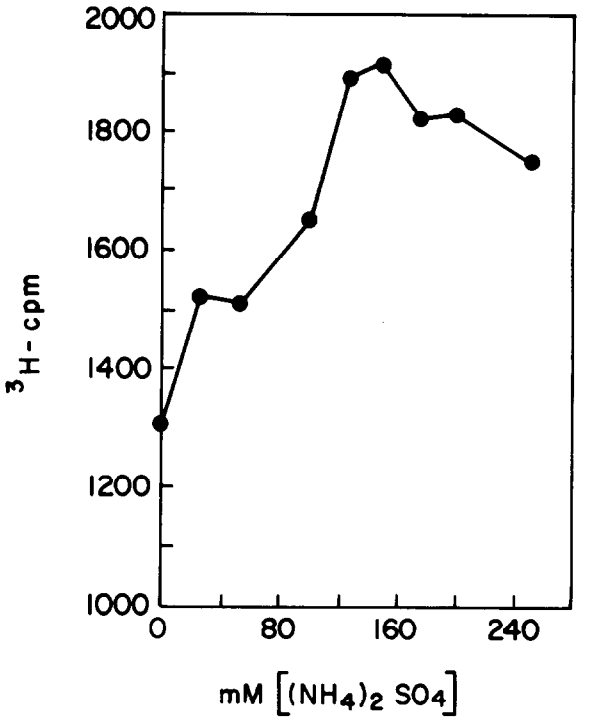

Fig.3. Effects of $(NH_4)_2SO_4$ concentration on RNA synthesis in isolated carrot nuclei. Nuclei containing 0 or 245 mM salt were mixed to yield the final concentrations indicated.

3. RESULTS

The rate of RNA synthesis at 29°C was proportional to the number of nuclei present in the reaction mixture (fig.1). Under optimum conditions the maximum rate of incorporation was 1.71 pmol UMP . μg DNA (nuclear)$^{-1}$. min^{-1} and was linear for ~50 min. Recovery of radioactivity was at best 0.45% of input.

The divalent ion optimal concentrations were determined for transcriptional activity in the isolated nuclei. The profiles for Mn^{2+} and Mg^{2+} in fig.2 show rather narrow optima. The activity is increased up to 35% for Mn^{2+} at 2 mM and 71% at 15 mM.

The optimum concentration of $(NH_4)_2SO_4$ for transcriptional activity was determined to be ~150 mM. There is a very broad optimum range, yet incorporation of [^{3}H]UTP is increased by 46% at this concentration (fig.3).

The incorporation of [^{3}H]UTP into acid-insoluble products was inhibited by [α]-amanitin. At 4 μg/ml, [α]-amanitin polymerization was inhibited by 21% (table 1). This effect was likely to be due to inhibition of RNA polymerase II activity [2,3,5]. No additional inhibition was seen at up to 400 μg [α]-amanitin/ml in these carrot nuclei.

Incorporated acid-insoluble radioactivity was sensitive to degradation by exogenously added RNase. Table 1 shown that ≥ 54% of the counts were rendered acid-soluble after RNase treatment of lysed nuclei after incorporation. Also, heat-shocked nuclei did not retain the ability to incorporate [^{3}H]UTP.

Table 1

Effects of inhibitors and exogenous RNase on in vitro RNA synthesis by isolated carrot nuclei

Sample	cpm	pmol UMP . μg DNA^{-1} . min^{-1}	% Control
Control	1237	1.60	100
RNase[a]	545	0.71	44
65°C/15 min[b]	172	0.22	14
Control	1140	1.42	100
[α]-Amanitin (4 μg/ml)	901	1.16	79

[a] RNase was at 100 μg/ml for 1 h at 37°C after lysis of nuclei with 0.1% (w/v) sarkosyl after incorporation

[b] Nuclei were heated to 65°C for 15 min before adding radioactivity

Nuclei were incubated with [^{3}H]UTP in standard reaction mixture containing 2 mM $MnCl_2$, 15 mM $MgCl_2$ and 150 mM $(NH_4)_2SO_4$. Nuclei (4.2 × 10^5/assay) were incubated at 29°C for 40 min. Results are the average of duplicate samples

To test for endogenous RNase activity, nuclei were incubated with 28 S r[^{14}C]RNA from bovine testes. Fig.4 shows the pattern of sedimentation of the unreacted 28 S rRNA. When this RNA was incubated with carrot nuclei, the distribution of radioactivity in the gradient had a much reduced *S*-value. There was a very broad peak up to the top of the gradient to ~ 4–5 S. Total recovery of the gradients was nearly identical and because all counts here represented trichloroacetic acid-precipitable counts, it is expected that this nuclease activity is mostly of the endonuclease type. Much of this nuclease activity could be inhibited by including exogenous double-stranded DNA with the 28 S rRNA and nuclei (fig.4). There was an obvious peak in the region to which the undigested 28 S rRNA marker sedimented.

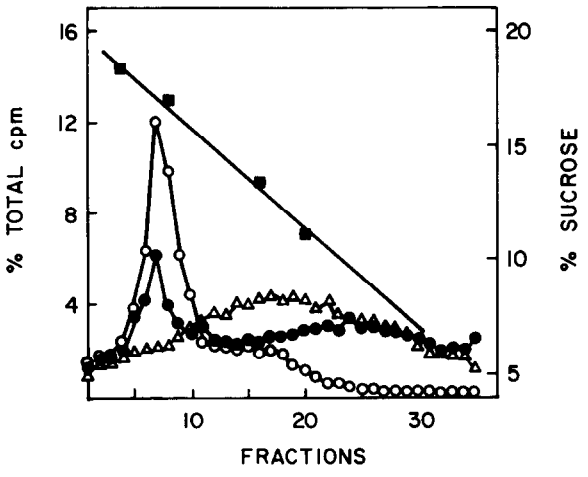

Fig.4. Sucrose gradient analysis of 28 S r[^{14}C]RNA mixed with carrot nuclei: assay for nuclease activity. Bovine testes 28 S rRNA without nuclei added (○——○), and with nuclei added (△——△). Bovine testes 28 S rRNA with nuclei salmon sperm DNA 20 μg/sample (•——•). All counts are trichloroacetic acid-insoluble; sedimentation is from right to left.

S-Values of in vitro synthesized RNA was determined by neutral sucrose gradient centrifugation. RNA up to 18 S was detected with the majority (80%) of the material sedimenting as a 2–7 S species (fig.5). The remainder of the radioactivity was detected as a > 8 S species. No clearly distinct high *S*-value species (> 10 S) was detected even after lysis of nuclei directly on top of the sucrose gradient. Direct lysis circumvented the possibility of degradation during the extraction procedure.

Attempts to inhibit ribonuclease activity by the vanadylribonucleoside complex (BRL) were unsuccessful. In fact, at 10 mM this complex inhibited [^{3}H]UTP incorporation by 83%, possibly by blocking the polymerases.

Exogenously added double-stranded salmon sperm DNA markedly stimulated acid-precipitable

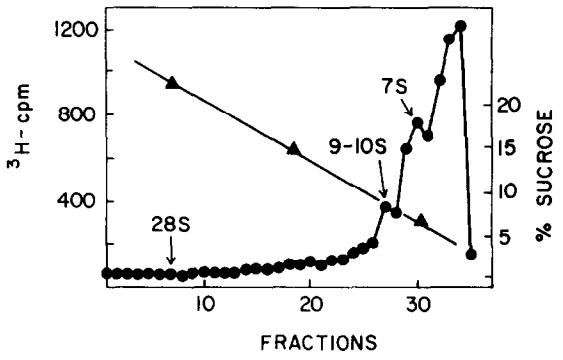

Fig.5. Sucrose gradient analysis of in vitro synthesized RNA from isolated carrot nuclei. Extracted RNA was heat denatured and layered onto sucrose gradients containing sarkosyl. All counts are trichloroacetic acid-insoluble; sedimentation is from right to left.

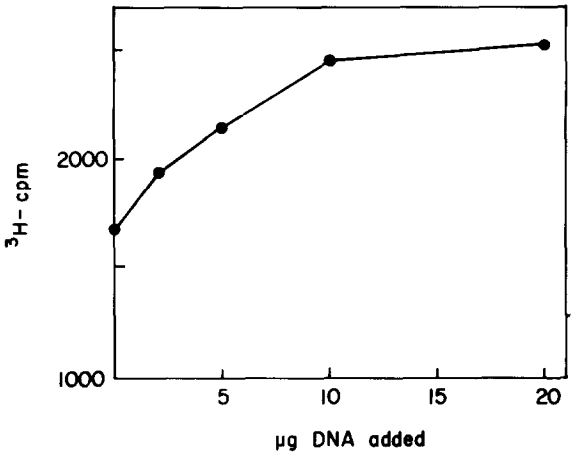

Fig.6. Exogenous DNA stimulation of RNA synthesis in isolated carrot nuclei. Nuclei (4.2 × 10^5/assay) were incubated for 30 min at 29°C with various concentrations of DNA. Results are the average of duplicate samples.

counts recovered in this nuclear transcription system. The greatest stimulation (51% increase) and saturation occurred at 20 μg added DNA/assay mixture of 400 000 nuclei (fig.6). Determined by microscopic observation, the nuclei remained intact and ellipsoidal even in the presence of 100 μg added DNA.

Measures of the amount of polyadenylated RNA made in vitro were determined by oligo(dT)–cellulose column chromatography. The pattern of elution was quite similar to that of in vivo synthesized RNA (not shown). In the in vitro sample, the polyadenylated RNA represents only 0.4% of the total added acid-insoluble RNA counts, whereas the in vivo sample contains 1–2% adenylated RNA.

4. DISCUSSION

A rapid procedure for isolating nuclei from carrot suspension culture is described. Starting with 16 ml PCV of logarithmically growing cells, up to 1.4 × 10^8 nuclei could be isolated from the Percoll gradients. These carrot nuclei were found to be active in transcription and the maximum rate of UTP incorporation into RNA was found to be 1.71 pmol UTP . μg DNA $^{-1}$. min $^{-1}$. This represents ~300-fold increase over what has been measured in wheat embryo nuclei [2,3], yet > 15-fold less than is seen in nuclei from the garden pea [4]. This rate is, however, comparable to the rate seen in corn nuclei [17]. The differences seen in the various materials listed above may be a reflection of differences in metabolic activity. Carrot suspension culture cells with a doubling time of < 2.5 days are expected to be more active than dormant, ungerminated wheat seeds.

Several reports have been published concerning characterization of the optimum salt conditions for RNA synthesis in isolated plant nuclei [2,3,6]. We have found divalent cation optima similar to those reported in tobacco [18] and wheat [2,3]. We all record a 15 mM [Mg^{2+}] optimum. The monovalent cation optimum in carrot nuclei was found to have a broad maximum with a peak value of ~ 150 mM. This is in rough agreement with the optimum of tobacco [19] and higher than the 25–100 mM optimum seen in wheat nuclei [2,3].

The degree of inhibition of transcription in carrot nuclei afforded by [α]-amanitin is less than that seen in other plant in vitro systems. In wheat [11,12], tobacco [3], and turnip [6], 4 μg/ml [α]-amanitin reduces transcriptional activity to 30–50% of control levels. It is possible that the system studied in particular and the amount of nuclear-associated RNA polymerase II determine sensitivities.

Logarithmic carrot cell nuclei respond to stimulation by exogenously added DNA. Two other plant nuclei in vitro transcription systems have been reported demonstrating that added exogenous DNA affects the RNA polymerase activity. In petunia

nuclei, transcripts directed by exogenous DNA are synthesized [7]. In tobacco, up to 8-fold increases of acid-insoluble counts in nuclei incubated with 100 μg DNA were shown [8]. Although our carrot nuclei transcription system responds to exogenous DNA, the increases are much less than reported in tobacco. We see typically a 50% increase in UTP incorporation. Whether or not this is true stimulation and a measure of free RNA polymerase or a protective effect against nuclease is not known. However, there is a notable protective effect afforded by exogenous DNA added to nuclei incubated with 28 S rRNA from bovine testes.

Several previous reports of RNA synthesis in isolated nuclei from plants have shown that little, if any, RNA is detected > 20 S. In pea shoots [20], pea leaves [4], and in corn shoots [1], the majority of the RNA synthesized is 4–15 S with no distinct size classes evident. This was also found to be true of nuclei isolated from carrot suspension cultures. Only the wheat embryo nuclei system [2,3] has shown clearly evident size classes of RNA synthesized and up to 28 S RNA being made.

In conclusion, we have shown that nuclei can be rapidly isolated from carrot suspension cultures. Microscopically, the nuclei appear intact and free from cytoplasmic debris. Significant amounts of messenger RNA length transcripts (9–10 S) and polyadenylated RNA are synthesized and the systems respond to added exogenous DNA.

This latter observation confirms the results in [8]. However, no estimate of the ribonuclease content of the nuclei preparation in [8] was provided. Our results show that exogenously added DNA was able to inhibit the ribonuclease activity associated with carrot nuclei. Therefore, it is not all certain that a stimulatory effect of DNA reflects bona fide transcription of the added template and/or amount of free RNA polymerase in nuclei. Experiments with isolated plant chromatin and cloned eukaryotic promoters should clarify these points and are in progress in our laboratory.

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