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Externally added DNA molecules support initiation of transcription in isolated nuclei from petunia

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Nuclei isolated from protoplasts transfected with the pUC8CaMVCAT and pDO432 plasmids were able to support, in run off experiments, the synthesis of specific transcripts as was evident from analysis by dot blot hybridization. Also the addition of the above plasmids to nuclei, prepared from non-transfected protoplasts, supported the synthesis of specific transcripts. Dot blot analysis showed that most of the transcripts obtained were complementary to the relevant gene sequences. α-Amanitin, at concentrations which are known to block the activity of RNA polymerase II, significantly inhibited the synthesis of specific transcripts by the isolated nuclei. The transcription activity was found to be predominantly associated with the nuclear fraction while the transcription products (RNA molecules) appeared in the supernatant obtained following sedimentation of the nuclei.

Chloramphenicol acetyltransferase gene; DNA topology; Transcription initiation; Isolated nucleus; Luciferase gene

1. INTRODUCTION

During the last few years, in vitro transcription systems, especially those obtained from animal cells, have been used as an experimental tool to investigate questions relating to control of gene expression [1,2]. With the aid of such systems, it has been demonstrated that specific DNA sequences and protein molecules can significantly modulate gene expression. Fractionation of an in vitro transcription system from HeLa cells allowed the purification and identification of several transcription factors and the assessment of their role in gene expression [3–5].

While much knowledge has been accumulated regarding the role of specific transcription factors and DNA regulatory elements in controlling gene expression in animal cells, very little is known about such factors in plant cells. Recently, an in vitro transcription system from wheat germ nuclei was established [6,7]. Using this system, initiation of accurate and specific transcription processes were demonstrated [7,8]. However, these in vitro systems cannot be used to study questions related to the regulation of expression of genes located in the chromosome and the effect of chromatin on their activity [5]. Evidently, such questions may be studied in transcriptionally active isolatec nuclei in which the natural environment and the organization of the genes in the chromosome is preserved [8]. Isolated nuclei can also serve as an excellent experimental tool for the elucidation of the mechanism by which biologically active macromolecules move across the nuclear envelope [9,10].

Nuclei purified from animal cells and lower eucaryotic cells have been shown to accurately transcribe r-RNA and t-RNA genes [11,12]. In plant cells, it was shown that isolated nuclei from wheat germ embryos were also able to support initiation of transcription catalyzed by RNA polymerase III [13]. Recently, initiation of transcription by RNA polymerase II was observed in nuclei of animal cells. This was inferred from experiments showing that the addition of cloned estrogen receptor to nuclei from frog liver cells induced accurate initiation of transcription of the vitellogenic gene [14].

In the present work, it is shown that externally added DNA can serve as a template for transcription in nuclei isolated from petunia protoplasts. Transcription was inhibited by the addition of α-amanitin, indicating that it was catalyzed by RNA polymerase II.

2. MATERIALS AND METHODS

2.1. *Plasmids*

The plasmid pUC8CaMVCAT was a generous gift from Dr V. Walbot (Department of Biological Science, Stanford University). This plasmid consists of the cauliflower mosaic virus (CaMV) 35S promoter, the coding region of the bacterial chloramphenicol acetyltransferase (CAT) gene and the polyadenylation site of the nopaline synthase (NOS) gene (M. Fromm, personal communication, and [15]).

The plasmid pDO432 was a generous gift from Dr S.H. Howell (University of California, San Diego). This plasmid contains the coding region of the firefly luciferase gene linked to the CaMV 35S promoter at the 5′ end and to the NOS polyadenylation region at the 3′ end [16].

The plasmid pSS15 was a generous gift from Dr R. Fluhr (Depart-

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ment of Plant Genetics, Weizmann Institute of Science, Rehovot, Israel). This plasmid contains cDNA of the small subunit of ribulose-1,5-bisphosphate carboxylase (ss-Rubisco) gene which was cloned from pea [17].

The plasmids were propagated and isolated as previously described [18].

2.2. *Preparation of protoplasts*

Protoplasts of *Petunia hybrida* (line 3704) were prepared from three-day-old cells as described before [18,19].

2.3. *Isolation of nuclei*

Nuclei were isolated essentially as described by Saxena et al. [20] with the following modifications: petunia protoplasts were deplasmoliesed (10^7 protoplasts) in 100 ml of nuclei-isolation buffer (NIB) [20], containing 0.01% Triton X-100, for 5 min and then disrupted by passage through 25 gauge needles. For this purpose, we constructed a special cylinder composed of 3 successive compartments each with 12 needles of 25 gauge. Passage of the desplasmoliesed protoplasts through the needles was driven by suction with vacuum and the suspensions obtained were then filtered through 3 layers of Miracloth and through nylon filters of 20 μm diameter/mesh pore size.

The filtered nuclei were washed with NIB (without Triton X-100 and then subjected to further purification on a Percoll column as described by Chappel and Hahlbrock [21]. The purified nuclei were washed twice with 5 vols of NIB (without Triton X-100) to remove residual Percoll and the pellet obtained was suspended in 1–2 ml of 20 mM Hepes (pH 7.2), 5 mM $MgCl_2$, 50% (v/v) glycerol and 5 mM β-mercaptoethanol. The nuclei were stored at −70°C until use.

The number of nuclei was estimated by microscopic examination using a hemocytometer, after staining with phenosafranine. The average yield of nuclei varied between 70 and 90%, taking as 100% the number of the protoplasts used for the preparation.

2.4. *Determination of transcription in isolated nuclei and isolation of RNA (*run-off experiments*)*

Aliquots of 5×10^6 nuclei were washed twice with 10 vols of the transcription buffer (TB) which contained (in mM): 17 Hepes (pH 7.9), 14 glycerol, 5 dithiothreitol, 0.1 EDTA, 55 KCl, 7 $MgCl_2$, 166 $(NH_4)_2SO_4$, 70 sucrose and 0.5 ATP, CTP and GTP. The nuclei were sedimented in Eppendorf tubes at $8000 \times g$ for 4 min, and resuspended with 90 μl of TB containing 50 units RNasin (Promega) and 100 μCi of [α-^{32}P]UTP (3000 Ci/mmol, Amersham). Transcription was carried out at 28°C for 45 min unless otherwise indicated. When α-amanitin was used, it was supplemented at a final concentration of 5 μg/ml. The transcription reaction was terminated by the addition of 30 units of DNase I (Promega) followed by incubation at 37°C for 15 min. At the end of the incubation period, the reaction mixture was diluted (v/v) with H_2O and then extracted with an equal volume of phenol. Non-incorporated nucleotides were separated from the RNA by a Sephadex G-50 column [20]. The RNA obtained was kept at −70°C until use.

For the kinetic studies, 5-μl aliquots of the above reaction mixture were withdrawn at various incubation times. The reaction was stopped by DNase I treatment as above, diluted with 10 vols of H_2O and extracted with phenol. For determination of radioactivity, aliquots of 10 μl were loaded on DE-81 discs (Whatman). Free nucleotides were removed by repeated washings with 0.5 M phosphate buffer and the total radioactivity in the RNA was measured by a liquid scintillation counter.

2.5. *Dot blot hybridization experiments*

Denatured DNA fragments, 1–2 μg each, were attached to nitrocellulose filters according to Davis et al. [22]. The fragments which were used as probes for the identification of specific run-off transcripts were obtained by digestion of pUC8CaMVCAT and pDO432 with *Hin*dIII and *Bam*HI, respectively [16,23]. The DNA fragments containing the CAT and the Luciferase gene sequences were separated by agarose gel electrophoresis and obtained by electroelution.

Nitrocellulose filters with the immobilized DNA probes were prehybridized overnight to heat-denatured calf thymus DNA (500 μg/ml) at 42°C in the presence of 50% formamide, 0.75 M NaCl, 0.115 M Na-citrate, 0.2% SDS, 5% Dextran sulfate and 0.04% of each, bovine serum albumin, polyvinylpyrolidane and Ficoll. Hybridization to ^{32}P-labelled RNAs (2–3 × 10^6 cpm, denatured by heating for 10 min at 60°C) was performed for 20 h at 42°C in the same solution. The relative amounts of the hybridized transcripts were quantitated from the autoradiogram by a Soft Laser scanning densitometer model SL-TRFF Biomed Instruments.

2.6. *Transfection of protoplasts with plasmid DNA*

Introduction of the plasmid pUC8CaMVCAT into plant protoplasts was performed with the aid of polyethylene glycol as described before [18].

3. RESULTS

3.1. *Externally added DNA as a template for transcription processes in isolated petunia nuclei*

Petunia nuclei prepared by the procedure described in section 2, were found intact and undamaged by electron microscopy studies (not shown). Quantitative studies (Fig. 1) clearly show that these nuclei were highly active in transcription processes as is evident from their efficient ability to incorporate [^{32}P]UMP into polyribonucleotides. The transcription activity of the isolated nuclei appear to be linear up to 70 min at 28°C (Fig. 1). At longer incubation periods, a decrease in the transcription rate was observed (not shown).

The ability of the isolated nuclei to use externally added DNA molecules as a template for transcription was studied by the use of two different plasmids, namely pUC8CaMVCAT and pDO432. Dot blot analysis was used to characterize labelled RNA molecules which

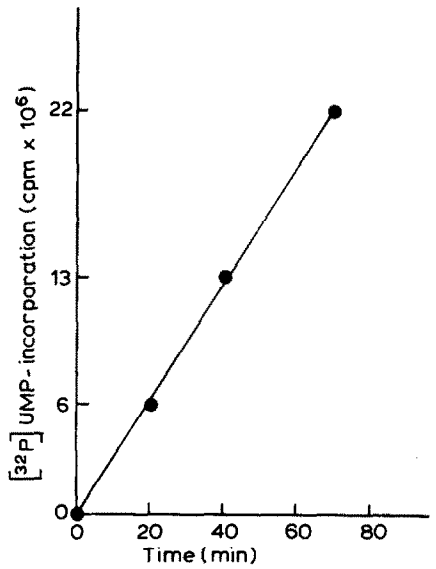

Fig. 1. Transcription activity of isolated petunia nuclei: kinetic studies. Incorporation of [^{32}P]UMP into RNA was performed and determined as described in section 2. The results given are an average of 3 independent experiments.

were synthesized ('run-off experiment') in nuclei incubated in the presence of the above-added plasmids. Our results show that such labelled RNA molecules hybridized predominantly to DNA probes possessing the CAT gene sequences (Fig. 2C-I and III). Significantly lower amounts of the same ^{32}P-labelled RNA preparation hybridized to DNA fragments lacking the CAT gene sequences (Fig. 2C-II) and no hybridization was observed when the Luciferase gene (Fig. 2C-IV) or DNA from calf thymus (Fig. 2C-V) were used as probes.

Transfection of petunia plant protoplasts with the plasmid pUC8CaMVCAT results in transient expression of the CAT gene [18,24]. In the present experiments, nuclei were isolated from protoplasts 4 h post-transfection, a time at which the highest levels of the transfected pUC8CaMVCAT plasmid were found to be associated with the nuclei fraction [18]. Our results (Fig. 2A) show that the pUC8CaMVCAT plasmid serves as a template for transcription in these isolated nuclei. This can be inferred from the hybridization pattern (Fig. 2A) which indicates synthesis of specific transcripts in such nuclei. Thus, it appears that the hybridization pattern of ^{32}P-RNA that was extracted either from nuclei incubated with externally added pUC8CaMVCAT or from nuclei obtained from transfected cells, was identical (compare Fig. 2A and C).

The specificity of the transcription process observed in the isolated nuclei is further emphasized by the results depicted in Fig. 2B and D. RNA molecules which were synthesized either by nuclei obtained from mock transfected protoplasts (Fig. 2B) or by nuclei incubated in the absence of externally added plasmid (Fig. 2D) failed to hybridize to the CAT gene sequences. On the other hand, RNA molecules extracted from both systems, hybridized to DNA sequences of the ss-Rubisco gene (Fig. 2A–D, VI). This gene is constitutively expressed by these cells during growth under continuous illumination [25]. These results further support our view that the nuclei preparations obtained in the present work were able to derive specific transcription processes. Run-off transcripts which were hydrolyzed with NaOH failed to show any hybridization capability (Fig. 2E), unequivocally proving that the dots obtained in Fig. 2 indeed resulted from hybridization of newly synthesized RNA molecules.

The results in Fig. 3A show that specific transcripts were also synthesized by the isolated nuclei following the addition of the plasmid pDO432. The synthesis of these transcripts (Fig. 3A) as well as those obtained following the addition of pUC8CaMVCAT (Fig. 3B) was dependent on the amount of the DNA added to the nuclei. In both cases, maximum activity was reached following the addition of 10–14 μg DNA/5 $\times$ 10^6 nuclei (not shown).

The results in Fig. 3C show that the addition of α-amanitin at a concentration which is known to block

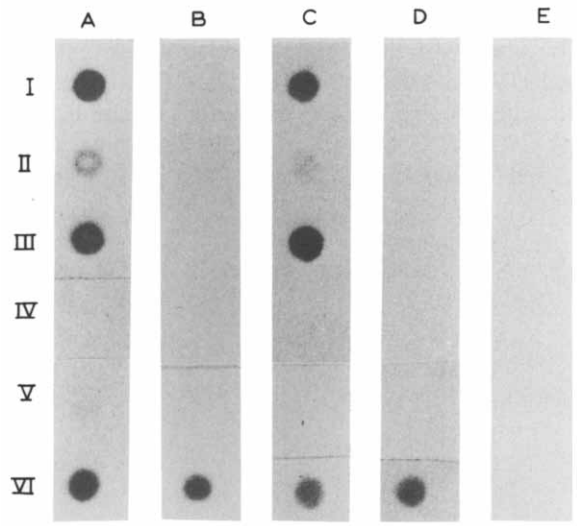

Fig. 2. Synthesis of specific transcripts in isolated nuclei as demonstrated by dot blot hybridization. ^{32}P-labelled transcripts were extracted from nuclei prepared from protoplasts which were pretransfected with pUC8CaMVCAT (A), calf thymus DNA (mock transfection) (B), or from nuclei of untransfected protoplasts which have been incubated in the presence (C, E) or in the absence (D) of pUC8CaMVCAT. In (E), the transcripts obtained were treated with 0.1 M NaOH and then neutralized to pH 7.5. The RNA extracts from the above systems were hybridized to the following immobilized DNA probes: (I) CAT gene sequence; (II) the pUC8CaMVCAT plasmid DNA from which the CAT gene sequence was omitted; (III) pUC8CaMVCAT; (IV) Luciferase gene sequence; (V) calf thymus DNA; (VI) pSS15. All other experimental conditions are as described in section 2.

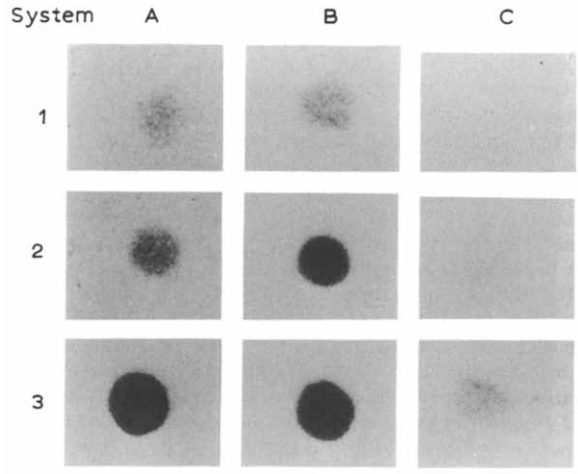

Fig. 3. Transcription of exogenously added DNA in isolated nuclei: effect of DNA concentrations and sensitivity to α-amanitin. Isolated nuclei were incubated with the following DNA plasmids: (A) pDO432; (B) pUC8CaMVCAT; (C) as in B, but in the presence of α-amanitin (5 μg/ml). DNA concentrations in systems 1, 2 and 3 were (in μg) 2, 4 and 7, respectively. RNA molecules extracted from each system (A–C) were hybridized to the same DNA plasmids which were used in the transcription system. All other experimental conditions are as described in section 2.

Table I

Exogenously added DNA as a template for specific transcription in isolated nuclei: effect of DNA topology

	Plasmid added (μg)	Relative amount of transcript (%)	
		pUC8CaMVCAT	pDO432
Linear plasmids	7	100	100
	4	84	52
	2	24	9
Supercoiled plasmids	7	58	20
	4	20	6
	2	5	1

Various amounts of the linear and supercoiled forms of pUC8CaMVCAT and pDO432 were incubated with isolated nuclei as described in section 2. Quantitative estimation of RNA is as described in Fig. 5

the activity of RNA polymerase II [26], significantly inhibited the appearance of specific transcripts in the isolated nuclei.

Previously, we have reported that the linear forms of the plasmids pUC8CaMVCAT and pDO432 support higher levels of transient gene expression in petunia or tobacco protoplasts than the supercoiled form of these plasmids [18]. The results in Table I show that also in isolated nuclei the linear form of the above two plasmids serves as a better template for transcription. As can be seen (Table I), incubation of isolated nuclei with linearized plasmids resulted in the synthesis of 5–10-fold more transcripts than incubation of these nuclei with the supercoiled structure of the same plasmids. These results were observed at different concentrations of the above DNA constructs (Table I).

3.2. *Localization of the transcription activity and of the newly synthesized transcripts*

The localization of the transcription activity and the distribution of the newly synthesized transcripts was determined in the supernatant and the pellet obtained following centrifugation of the nuclei. Quantitative estimation revealed that most of the specific transcription activity observed was associated with the nuclear pellet (compare Fig.4A and C). Very little, if any, transcription activity was detected in the supernatant, indicating that transcription was supported by the nuclei and not by a soluble fraction. Furthermore, our results show that preincubation of the isolated nuclei in the transcription buffer (without UTP) for 30 min at 28°C (Fig. 4C) did not affect their activity, indicating that probably not many of the factors needed for transcription were released from the nuclei during the preincubation period. On the other hand, the specific transcripts which were synthesized by the isolated nuclei, were present mainly in the supernatant obtained following sedimentation of the nuclei (Fig. 4B). Uptake

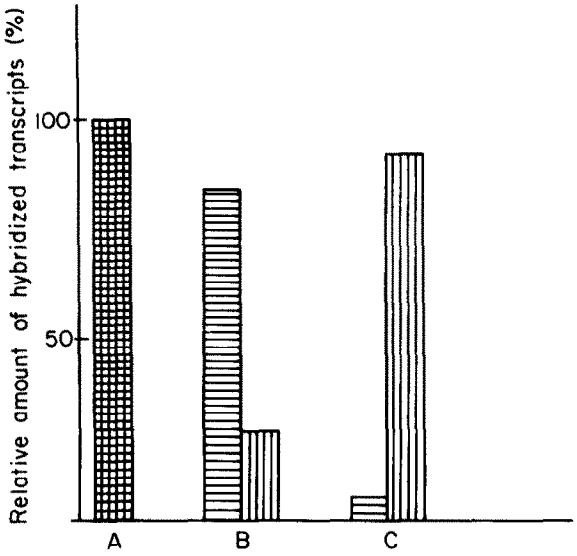

Fig. 4. Association of the transcription activity with the nuclei fraction and release of the transcripts to the supernatant. (A) Isolated nuclei were incubated with pUC8CaMVCAT (4 μg), and then RNA was extracted as described in section 2. The specific transcripts obtained were assayed by dot blot hybridization and their relative amounts were determined as described in section 2. (B) For determination of RNA distribution, nuclei were incubated with pUC8CaMVCAT and before the RNA was extracted, the nuclei were centrifuged (8000 × g) and the pellet obtained was resuspended in 90 μl of TB (see section 2). RNA was extracted simultaneously from the pellet (vertical lines) and the supernatant (horizontal lines). The RNA was quantitatively estimated as in (A). (C) Before addition of the DNA plasmid, the isolated nuclei were incubated for 30 min at 28°C in 90 μl TB, then the nuclei suspension was centrifuged (8000 × g) and the pellet obtained was resuspended in 90 μl of TB. In order to study the localization of the transcription activity, the plasmid pUC8CaMVCAT (4 μg) and [^{32}P]UTP were added to the resuspended pellet (vertical lines) and to the supernatant (horizontal lines). Following incubation for 45 min at 28°C, RNA was extracted. The appearance of specific transcripts was analyzed and quantitatively estimated as in (A). Results are given as percent of transcripts obtained, taking as 100% the amount of specific transcript obtained in (A).

of DNA molecules into nuclei isolated from yeasts have been observed before and reported recently [27].

4. DISCUSSION

The most interesting finding of the present work is the demonstration that externally added DNA molecules can serve as a template for transcription processes in isolated nuclei. Appearance of specific transcripts is evident from dot blot analysis which showed that DNA probes containing the CAT gene sequences were able to hybridize predominantly to RNA molecules synthesized by isolated nuclei when externally added pUC8CaMVCAT served as a template. The low hybridization levels, which were still observed with DNA probes lacking the CAT gene can be attributed to transcripts obtained from the vector sequences. This assumption, supported by our results showing that

DNA molecules of unrelated sequences such as those of the calf thymus or the Luciferase gene failed to show any hybridization under the stringent conditions used.

Similarly, RNA extracts obtained from nuclei incubated with the plasmid pDO432 hybridized mainly to DNA probes containing the Luciferase gene sequences, thus strengthening the view that externally added DNA molecules can serve as a template for the synthesis of specific transcripts.

Transfection of petunia protoplasts with the plasmids pUC8CaMVCAT and pDO432 have been shown previously to induce the synthesis of the CAT and the Luciferase enzymes, respectively [18]. Our present results show that transcripts synthesized by nuclei of transfected protoplasts, or by nuclei to which the above two plasmids were externally added, were hybridized in a similar manner to various DNA probes. Northern blot analysis of the same extracts also clearly indicated the appearance of specific transcripts of different molecular weights, ranging from 200 to 1500 bp (not shown).

It is noteworthy that the plasmids used in the present work do not integrate into the chromosomal DNA but remain as free molecules within nuclei of the transfected cells [6,18,24]. In the present study the transcription activity was found to be mainly associated with the isolated nuclei and was preserved within the nuclei even after 30 min incubation at 28°C, indicating that no massive loss of transcription factors occurred under these conditions. On the other hand, most of the newly synthesized transcripts were present in the supernatant fraction. The difference in the localization between the newly synthesized RNA molecules (supernatant) and the transcription activity (pellet) may indicate that the transcripts formed are exported from the nuclei by a mechanism which may be similar to the one suggested to operate in intact living cells, namely through the nuclear envelope pores [10,28]. It is possible that also the externally added DNA penetrates into the nuclei via a specific and regulated mechanism. Thus, the present isolated nuclei, besides being a convenient tool to study regulation of transcription processes, may also offer a model to study movement of nucleic acids into the nuclei compartment. Evidently, more studies are required to confirm this possibility.

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