STUDIES ON THE CONTROL MECHANISM OF RNA POLYMERASE I ACTIVITY IN HIGHER PLANTS[1]

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Abstracts: In order to study the relationship between ribosomal function and rRNA synthesis, three-day old soybean seedlings were immersed in distilled water and bubbled with N_2 gas for various durations.

The brief N_2-gas treatment caused a decrement of the polyribosomal level in the meristematic region and a reassociation of monomeric ribosomes to polyribosomes by aeration as previously reported. The treatment with N_2 gas also significantly decreased the RNA polymerase I activity as expressed in the isolated nuclei. Using a three-hour treatment, the RNA polymerase I activity expressed in the isolated nuclei dropped to 35% of the initial activity. However after solubilization of the enzyme from the isolated nuclei and then fractionation on DEAE-cellulose column, the RNA polymerase I activity was similar to that from the control. After two-hour treatment of N_2 gas and then aeration for two hours, the RNA polymerase activity in meristematic region return to its original level. These results indicated that there is a close relationship between the polyribosomal level (protein synthesis) and the rRNA synthesis.

When ppGpp (one of the stringent factors in *E. coli*) was added to the *in vitro* synthetic reaction mixtures, even at a half concentraction of the GTP nucleotide substrate, used in the assay, there was no inhibition of RNA synthesis.

SDS-gel analysis of the nuclear proteins by electrophoresis showed no distinct differences between the N_2-gas treated tissues and the control tissues. The regulation of rRNA synthesis in the meristematic region of soybean hypocotyl needs further study.

INTRODUCTION

In eukaryotic cells, genetic materials are present in nucleus, chloroplast, and mitochondria. The first step in controlling gene expression lies at the transcription on RNA from its DNA template, and the second step lies in controlling gene expression at the translation of proteins (or enzymes) from its RNA message. The genes which code for ribosomal RNA are associated with the nucleolus, which is devoted to the synthesis, the maturation of the ribosomal RNA precursor molecules and the assembly of the ribosomes. RNA polymerase I is also localized in the nucleolus and is engaged in transcription of the precursor of rRNA. RNA polymerase II is present in the nucleoplasm and is engaged in the transcription of the precursor of messenger RNA[17]. After auxin treatment, cell division is inhibited in the meristematic zone and cell elongation ceases in the meristematic and elongation zones[11]; radial enlargement of the cells occur in the elongation and mature zones. The abnormal proliferation of the mature hypocotyl

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Abberviations: bis: N, N′-methylene-bis-acrylamide; MES: 2 (N-morpholine) ethane sulfonic acid; ppGpp: Guanosine 5′-diphosphate 3′-diphosphate; rRNA: Ribosomal RNA; SDS: Sodium lauryl sulfate; TEMED: N, N, N′, N′-tetramethylethylene diamine; Tris: Tris (hydroxymethyl) amino methane.

is preceeded by a large increase in RNA, especially ribosomal RNA[4,10]. Nuclei isolated from the mature zone of auxin-treated hypocotyls have a much higher rate of RNA synthesis than the control nuclei[3,8]; this greater RNA synthetic activity is primarily due to a large and preferential increase (3- to 5-fold) in RNA polymerase I activity. RNA polymerase II activity only increased a few percent if at all[3,8].

When the meristematic zone of hypocotyls are excised and incubated in a phosphate buffer, there is a dramatic and rapid decrease in the activity of RNA polymerase I in the isolated nuclei with little or no change in the activity of RNA polymerase II. But the level of RNA polymerase I activity following solubilization from nuclei and fractionation on DEAE-cellulose was identical between zero time of control and 12-hr incubated tissues[12]. The level of polyribosomes drops rapidly from 75% to about 10% of the total ribosome population during 12 hrs-incubation of meristematic tissues[10]. A close coupling of rRNA synthesis and ribosome function has been the subject to intense study in bacteria[5] and to a lesser extent in some eukaryotic systems[6,7]. By using N_2 gas to induce the dissociation of polyribosomes into monomeric ribosomes and association of monomeric ribosomes to polyribosomes by aeration[11], the author has tried to study the coupling of rRNA synthesis and ribosome function and its control mechanism of RNA polymerase I activity in the soybean seedlings.

MATERIALS AND METHODS

Soybean seeds (*Glycine max* L. cv. Taita-Kaohsiung No. 3) were germinated in rolls of moist paper[9]. Three-day-old seedlings were immersed in distilled water and bubbled with either N_2 gas or air for various periods at room temperature.

Preparation of nuclei

The nuclei were isolated by a method of Chen *et al.*[3]. For RNA polymerase activity assay, the nuclear pellet was suspended in 1 M sucrose containing 25 mM MES-NaOH buffer (pH 6.0), 20 mM KCl, 30% glycerol, and 10 mM 2-mercaptoethanol.

Preparation of ribosomes

Two grams of tissues were homogenized with 7 ml of buffer A (containing 0.15 M tris-HCl at pH 8.5; 0.25 M sucrose; 50 mM KCl; and 10 mM 2-mercaptoethanol) with a Polytron PT 20 St setting at No. 10 for five seconds. The homogenate was filtered through two layers of cheesecloth and then two layers of miracloth. The filtrate was then centrifuged at 17,300×g for 25 min. The supernatant was layered over 5 ml of 65% (w/v) sucrose in buffer B (containing 40 mM tris-HCl at pH 8.5, 20 mM KCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol). The ribosomes were pelleted by centrifugation at 220,000×g for 90 min. in a type 65 rotor in a MSE Model 65 ultracentrifuge. The ribosome pellets were resuspended in buffer B and layered over linear sucrose gradient (15% to 60%, w/v) and centrifuged at 27,000 rpm for 3 hr in a MSE Model 65 SW 30 rotor. Gradients were scanned at 254 nm with an ISCO Model UA-4 absorbance monitor.

RNA polymerase assay

The RNA polymerase activities of the nuclear preparation were assayed at 28 C in a 0.25 ml reaction mixture containing 50 mM tris-HCl at pH 8.0, 10 mM dithiothreitol, 5 mM $MgCl_2$, 20% glycerol, 0.4 mM each of ATP, GTP, and CTP, and 0.02 mM ^{3}H-UTP (1 μCi). Other additions are described in the table legends. The reaction was terminated after 20 min of incubation by the addition of the 2 ml of 10% trichloroacetic acid containing 8 mM sodium pyrophosphate. The precipitate was collected on GF/C glass fiber disks, washed four times with 3 ml of 5% trichloroacetic acid and twice with 4 ml of 95% ethanol. The filters were dried under heat lamps and counted in a liquid scintillation spectrometer (Beckman IS-100C).

Solubilization of RNA polymerases from isolated nuclei and DEAE-cellulose chromatography

RNA polymerases were solubilized from isolated nuclei as described earlier[8]. The solubilized proteins were fractionated on DEAE-cellulose as described by Roeder and Rutter[17]

SDS-acrylamide gel electrophoresis

One part of nuclear suspension was mixed with one part of 0.125 M tris-HCl at pH 6.8, 4% SDS, 8 M urea, and 10% 2-mercaptoethanol. The mixture was heated in boiling water for 2 min. Resolving gel contained 0.375 M tris-HCl at pH 8.8, 0.1% SDS, 0.05% TEMED, 8.75% acrylamide and 0.32% bis. Stocking gel contained 0.125 M tris-HCl at pH 6.8, 0.1% SDS, 0.05% TEMED, 3.5% acrylamide and 0.093% bis. Electrode buffers contained 0.025 M tris base, 0.192 M glycine, pH 8.3, and 0.1% SDS. The DC power supply was used for electrophoresis. Samples were run into stocking gels at low current (about 2 mA per tube) for about one hour, then the current was increased to about 4 mA per tube. The gels were kept a constant voltage until the run was completed. Completion of the run was indicated by when the bromophenol blue dye came to 0.5 cm from the bottom of the gel. After the gels were removed from the bottom of the tube, they were immersed in a fixing solution (containing 50% isopropanol and 10% trichloroacetic acid) at 30 C for 30 min. The gels were then stained with 0.1% coomassie brilliant blue prepared in 25% isopropanol and 10% trichloroacetic acid, and destained with 10% acetic acid. The gels were scanned at 600 nm with ISCO gel scanner Model 659 with absorbance monitor (model UA-4).

RESULTS

Effect of N_2 gas treatment on polyribosomal level and RNA polymerase activities in isolated nuclei

When the intact seedlings dipped in distilled water were bubbled with N_2 gas for two hours and then the ribosomes were isolated from meristematic regions and fractionated on sucrose gradient, the results of the OD_{254} profiles of the ribosomes are shown in Fig. I. The N_2-gas treatment caused the dissociation of polyribosome level in the hook tissues as reported previously[14]. Similarly the N_2-gas treated intact seedlings were used for nuclei isolation and the assay of RNA polymerases in the isolated nuclei. The RNA polymerase activities in the isolated nuclei decreased considerably during anaerobic treatment. (Fig. 2 and Table 1). RNA polymerase I activity decreased more than RNA polymerase II activity. At the end of 4 hours treatment with N_2-gas, the RNA polymerase I activity in the isolated nuclei remained only 35% of the initial activity. When the RNA polymerases were solubilized from the isolated nuclei and then fractionated on DEAE-cellulose, the level of RNA polymerase I activity in the N_2-gas treated tissues were similar to the control tissues. (Fig. 3 and Fig. 4). There was much less change of RNA polymerase II activity during the N_2-gas treatment. After two-hour treatment of N_2-gas and then aeration for two hours, the RNA polymerase activity in meristematic region returned to its original level (Table I). These results indicated that there was a positive relationship between the polyribosomal level and the RNA polymerase I activity (Fig. I and Table I).

Effect of ppGpp on in vitro RNA synthetic activities in the isolated nuclei

When ppGpp was added to the RNA polymerase assay reaction mixtures at a concentration equal to as much as half the concentration of ppGpp substrates (0.2 mM), there was no inhibition on RNA polymerase I activity as expressed in the isolated nuclei. (Table 2).

Effect of N_2-gas treatment on nuclear proteins

Nuclear proteins when prepared from the nuclei of control and N_2-gas treated seedlings using the method described in Materials and Methods were compared in SDS-gel by electro-

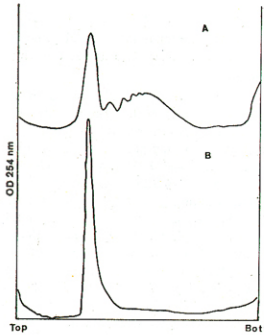

Fig. 1. The OD_{254} profiles of ribosomes on 15 to 60% sucrose density gradient. The ribosomes were isolated from the meristematic regions on control and N_2-gas treated seedlings and then layered on 15 to 60% (w/v) linear sucrose density gradient. After centrifugation at 27,000 rpm for 3 hrs in a MSE model 65 SW 30 rotor, gradients were fractionated and scanned at 254 nm with an ISCO model UA-4 absorbance monitor.
A: Ribosomes from the control tissues.
B: Ribosomes from the N_2-gas treated tissues.

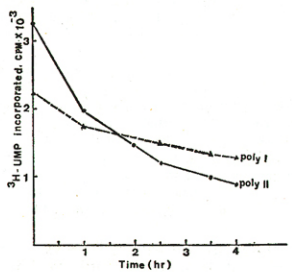

Fig. 2. In vitro RNA polymerase activities of isolated nuclei from meristematic regions of soybean hypocotyl under anaerobiosis. The three-day old seedlings were dipped in distilled water and then bubbled with N_2 gas for various durations. The nuclei were isolated from the hook tissues and then assayed for RNA polymerase activities. The assay procedures were the same as in the legends of Table 1.

phoretic analysis. The results of the electrophoresis are shown in Fig. 5. The electrophoretic patterns of nuclear proteins did not change qualitatively or quantitatively during the brief treatment (2 hr) of N_2-gas.

DISCUSSION

In our previous studies we have demonstrated that the correlation between the *in vivo* level of rRNA synthesis in various regions of control and auxin treated soybean seedlings and the level of RNA polymerase I (rRNA polymerase) as expressed in isolated nuclei[13]. Nuclei isolated from mature regions of auxin-treated seedlings has much higher rRNA synthetic activity than control nuclei and this greater activity is related primarily to a large and preferential increase in RNA polymerase I activity. Nuclei isolated from meristematic regions of auxin-treated seedlings or from 12-hr-incubated segments from the meristematic regions showed

Table 1. The influence of N_2-gas treatment on RNA polymerase activities in the hook tissues from soybean seedlings.

Nuclei source	cpm/4×10⁶ nuclei		Percentage (%)	
	Polymerase I	Polymerase II	Polymerase I	Polymerase II
Control	3,250	2,250	100	100
N_2 gas (2 hr)	1,500	1,600	46	71
N_2 gas (2 hr) then aeration (2 hr)	3,331	2,162	102	96

The isolated nuclei (4×10^6 nuclei) were assayed for RNA polymerase activities with ^{3}H-UTP as described in the Materials and Methods with the addition of ammonium sulfate. RNA polymerase I activity was based on the presence of 4 μg/ml α-amanitin at 50 mM $(NH_4)_2SO_4$. RNA polymerase II activity was determined by the difference of activity in the presence and absence of 4 μg/ml α-amanitin at 200 mM $(NH_4)_2SO_4$. The reaction mixtures were incubated at 28°C for 20 minutes.

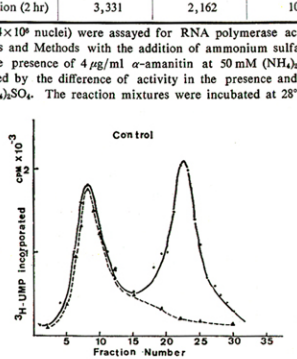

Fig. 3. DEAE-cellulose chromatography of RNA polymerase activities after solubilization from control soybean nuclei. Solubilized nuclear protein (12 A_{280} units) from control hooks were loaded onto DEAE-cellulose.
●——● No α-amanitin
▲- - -▲ 0.4 μg/ml of α-amanitin.

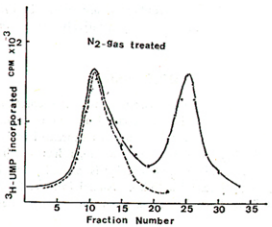

Fig. 4. DEAE-cellulose chromatatography of RNA polymerase activities after solubilization from N_2-gas treated nuclei. The nuclei were isolated from N_2-gas treated hooks and then the solubilized nuclear proteins (12 A_{280} units) were loaded onto DEAE-cellulose.
●——● No α-amanitin
▲---▲ 0.4 μg/ml of α-amanitin

Table 2. Effect of ppGpp on RNA polymerase activities in the isolated nuclei.

Concentration of ppGpp (mM)	RNA polymerase activity (cpm)	Concentration of ppGpp (mM)	RNA polymerase activity (cpm)
0	10,400	0.10	10,126
0.03	10,480	0.20	10,523

The nuclei were isolated from the hypocotyl of control, preincubated in ppGpp for 30 minutes at 0°C, and then assayed for its RNA polymerase activity. The isolated (8×10^6 nuclei) were sssayed in the reaction mixture containing 50 mM ammonium sulfate at 28°C for 20 minutes.

a marked decrease in the activity of RNA polymerase I as expressed in isolated nuclei. However, this decrease in activity was not due to the loss of the level of RNA polymerase I *per se* but was related to a supression of the enzyme activity since the level of the enzyme activity after solubilization was similar to control tissue. From above results showed there was a postive correlation between the level of RNA polymerase I activity expressed and the functional state of the protein synthetic apparatus as assayed by the level of polyribosomes in the meristematic region of soybean hypocotyls. Since the *in vitro* level of polyribosomes in soybean seedlings can be manupulated by anaerobiosis which causes rapidly dissociation of polyribosomes to monoribosomes and by anaerobiosis followed by aerobiosis which causes re-assembly of the monoribosomes to polyribosomes[14]. We use this manupulation to seek the strong relationship of the level of polyribosomes to the expression of RNA polymerase I activity. The RNA polymerase I activity dropped sharply to 62% of the initial level in a relatively short time of anaerobiosis whereas RNA polymerase II activity remained almost at the same level (only 29%) as the initial activity. Following anaerobiosis by aerobiosis, RNA polymerase I activity was recovered. This again demonstrates a very strong positive correlation between the level of RNA polymerase I activity expressed and the level of polyribosomes in the soybean tissues. The change of polymerase I activity during brief anaerobiosis and anaerobiosis followed by aerobiosis does not appear to be relatived to due to the changes of nuclear proteins as shown by SDS-gel electrophoretic analysis.

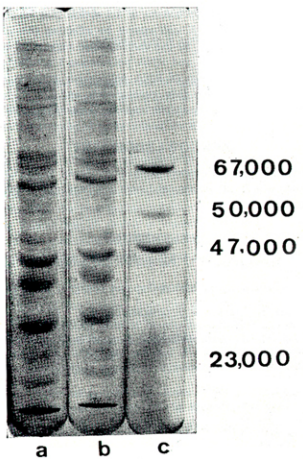

Fig. 5. Electrophoretic patterns of nuclear proteins in SDS-gel. Nuclear proteins from nuclei of soybean seedlings:
(a) under anaerobiosis for 2 hrs
(b) under aerobiosis for 2 hrs after 2 hrs of anaerobiosis.
(c) The standard molecular weight: Bovine serum albumin 67,000; γ-globulin large subunit 50,000; ovalbumin 47,000; γ-globulin small subunit 23,000.
The above nuclear proteins were on 8.75% SDS-gel. The electrophoretic patterns of control nuclear proteins were same as from under aerobiosis for 2 hrs after 2 hrs of anaerobiosis.

A close coupling of rRNA synthesis and ribosome function has been the subject of intense study in bacteria[5], and to a lesser extent in some eukaryotic systems[6,7]. The relaxed locus in *Escherichia coli* is the control element whose product, the stringent factor or the enzyme which catalyzes the formation of ppGpp[1], may serve to couple the synthesis of stable RNA species to protein synthesis. In this prokaryotic system, the accumulation of ppGpp or pppGpp which was synthesized by "idling" ribosomes and stringent factors[2] served to suppress the synthesis of stable RNA species[16,18]. But in this experiment ppGpp did not inhibit *in vitro* ^{3}H-UTP incorporation into RNA in isolated nuclei. So a different mechanism provably is operative in eukaryotes to couple ribosome function and rRNA synthesis. Recently, McMillian *et al.*[15] isolated a guanine-rich and low molecular weight of ribonucleic acid from a pathogenic fungus, *Histoplasma capsulatum*, they found this 4-6S ribonucleic acid could inhibit the

activity of RNA polymerase I. The mechanism of its action may be similar to the action of ppGpp to the RNA polymerase in the *E. coli* system by affecting the affinity of RNA polymerase I to the DNA template[12,13,15]. In the meristematic regions of soybean hypocotyls the ribosome function in protein synthesis and RNA polymerase I activity show a close coupling relationship. But the control mechanism of RNA polymerase activity in higher plants is still not fully understood. Whether or not this guanine-rich and low molecular weight of RNA regulates the RNA polymerase I activity in the soybean hypocotyl needs further study.

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