Phytochemistry, 1973, Vol. 12, pp. 2815 to 2820. Pergamon Press. Printed in England.

CHARACTERIZATION OF POLYSOMES AND INCORPORATION *IN VITRO* OF LEUCINE AND LYSINE IN NORMAL AND OPAQUE-2 *ZEA MAYS* ENDOSPERM DURING DEVELOPMENT

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(*Received* 23 *May* 1973. *Accepted* 18 *June* 1973)

Key Word Index—*Zea mays*, Graminae; maize; opaque-2; normal; lysine; leucine; regulatory control.

Abstract—Polysome preparations obtained from opaque-2 and normal maize endosperms during development did not show any significant difference in sedimentation coefficient or nucleotide composition. The pattern of incorporation *in vitro* of lysine and leucine, however, differed quite distinctly in these two preparations. During early stages of maturity the polysomes from opaque-2 incorporated substantially more lysine and less leucine as compared with those from normal maize. Although the trend was reversed at 25 days post-pollination, this did not result in any significant zein accumulation since very little total protein was synthesized after that stage in opaque-2 maize endosperm. It is, therefore, suggested that the opaque-2 gene exerts a regulatory control on *m*RNA synthesis, required for zein formation at early stages of maturation.

INTRODUCTION

IT IS NOW well established that opaque-2, a single recessive gene, is responsible for suppressing the synthesis of zein in maize endosperm. This in turn results in higher lysine and lower leucine concentrations in mature grains, since zein is extremely deficient in the former but rich in the latter.[1–4] Very little is known about the mechanism of suppression of zein synthesis by opaque-2 gene. It has been suggested that higher ribonuclease activities[5,6] in endosperm of developing grains of opaque-2 compared to those in normal maize may be responsible for the complete disappearance of RNA from the endosperm during penultimate stages of grain maturation.[7] The pattern of zein accumulation in opaque-2 is consistent with the suggestion that the opaque-2 gene is active only during the first 2–3 weeks after pollination.[3] Zein accumulation, however, continues in normal maize for a much longer period.

It is not known whether opaque-2 gene affects other parameters in later stages of grain development. The main object of the present study was to investigate differences, if any, in the physico-chemical and biological properties of polysomes from normal and opaque-2 endosperm during grain development. The capacity of the polysome preparations for incorporation *in vitro* of lysine and leucine was investigated. It is shown that major character-

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[6] WILSON, C. M. and ALEXANDER, P. E. (1967) *Science* **155**, 1575.
[7] MEHTA, S. L., SRIVASTAVA, K. N., MALI, P. C. and NAIK, M. S. (1972) *Phytochemistry* **11**, 937.

istic differences in the properties of polysomes do not exist. It is suggested that the effect of opaque-2 gene on the incorporation of leucine and lysine could be explained on the basis of regulatory control of *m*RNA synthesis.

RESULTS

Sedimentation Characteristics of Polysomes

The sedimentation coefficients for different peaks for polysomes from normal and opaque-2 endosperm before and after ribonuclease treatment at 15 and 25 days post-pollination are presented in Table 1. At 15 days post-pollination besides monomer, the 80*S*, polysomal particles up to tetramer were detected in normal and up to pentamer in opaque-2. At later stages of maturity the pentamer was not present in opaque-2. At both the stages dimers could not be detected. The absence of faster moving particles in polysome preparations at later stages may have been due to loss *in vivo* with age or degradation during isolation induced by high endogenous ribonuclease. This activity has been shown to be high despite the use of bentonite as an inhibitor.[7] Although the *S* values have not been extrapolated to infinite dilution they would not have changed much, since the concentration (1·5 mg/ml) used for the Schlieren runs was minimal. Treatment of polysomal preparations with 10 μg pancreatic ribonuclease for 5 min at 4° resulted in the disappearance of faster moving components and appearance of a small peak for dimer. This is in agreement with the results obtained for wheat.[8]

TABLE 1. SEDIMENTATION COEFFICIENTS OF NORMAL AND OPAQUE-2 POLYSOMES DURING GRAIN DEVELOPMENT

	15 days post-pollination		25 days post-pollination	
	Normal	Opaque-2	Normal	Opaque-2
Without RNase treatment				
	19	—	18	19
		70		
Ribosome monomer	80	83	82	82
Polysomes				
Dimer	—	—	—	—
Trimer	136	133	131	132
Tetramer	161	168	165	168
Pentamer	—	215	—	—
Hexamer				
After RNase treatment				
	20	17	20	19
Ribosome monomer	80	82	82	82
Dimer	100	101	102	101

Nucleotide Composition

Ribosomal RNA was prepared from normal and opaque-2 polysomes and nucleotide composition was determined by column chromatography on Dowex-formate form.

In order to ascertain the sequence and purity of the nucleotide fractions eluted from the columns, spectral readings at 250, 260, 280 and 290 nm were taken. The spectral ratios are shown in Table 2. The results were very close to calculated theoretical values. Hence the semi-micro method employed permitted sharp separation of mononucleotides.

[8] MEHTA, S. L., HADZIYEV, D. and ZALIK, S. (1968) *Biochim. Biophys. Acta* **169**, 381.

The results presented in Table 3 show the nucleotide composition of *r*RNA at 15 days post-pollination. At later stages the nucleotide composition was found to be similar. No differences were observed in the nucleotide composition of *r*RNA of opaque-2 and normal at different stages of maturity. The results obtained followed Chargaff's rule, i.e. the ratio of 6-amino/6-oxo bases was very close to 1·0 in both normal and opaque-2. The sum of Gp + Cp was greater than the sum of Ap + Up (Type GC). This is in agreement with the observation of higher GC content in plant *r*RNA.[9–11] Pseudouridylic acid was present to

TABLE 2. CHARACTERISTIC SPECTRAL READINGS OF *r*RNA NUCLEOTIDES

	Nucleotide (mixture of 2′ and 3′ acids)	Spectral ratios at pH 2 A_{250}/A_{260}	A_{280}/A_{260}	A_{290}/A_{260}
Normal	Ap	0·79	0·36	0·13
	Cp	0·51	1·79	1·27
	Up†	0·78	0·34	0·05
	Gp	1·07	0·69	0·32
Opaque-2	Ap	0·91	0·36	0·12
	Cp	0·52	1·77	1·25
	Up	0·79	0·33	0·05
	Gp	1·00	0·69	0·33

* Does not include the pseudouridylic acid: spectral ratios of ψ Up at pH 2; $A_{250}/A_{260} = 0{\cdot}76$; $A_{280}/A_{260} = 0{\cdot}38$; $A_{290}/A_{260} = 0{\cdot}06$.

the extent of 2·5 mol/100 mol of uridylic acid. This value is lower than the range of values obtained by other workers for *r*RNA from plants[10–12] and this could perhaps be due to the use of purified *r*RNA. The ratio of purine–pyrimidine nucleotides, a characteristic feature which may indicate specificity of RNA composition, was essentially the same for both normal and opaque-2 *r*RNA.

TABLE 3. NUCLEOTIDE COMPOSITION OF RIBOSOMAL RNA FROM NORMAL AND OPAQUE-2 ENDOSPERM AT 15 DAYS POST-POLLINATION

Nucleotide (mixture of 2 and 3′ acids)	Normal (mol %)	Opaque-2 (mol %)	Nucleotide (mixture of 2′ and 3′ acids)	Normal	Opaque-2
Cp	27·4	28·2	Purine–pyrimidine		
Ap	21·2	21·5	(Ap + Gp)/(Cp + Up + ψUp)	1·14	1·10
Up	18·8	19·0	6 amino/6-oxo bases		
ψUp	0·6	0·5	(Ap + Cp)/Gp + Up + ψUp)	0·95	0·99
Gp	32·0	30·8	(Gp + Cp)/(Ap + Up + ψUp)	1·46	1·44
			Ap/Cp	0·77	0·76

Amino acid Incorporation in Cell-free System

In order to study the influence of polysomes on the changed amino acid content of different protein fractions synthesized during grain development, the amino acid incor-

[9] BELOZERSKY, A. N. and SPIRIN, A. S. (1960) *Izv. Akad. Nauk. SSSR. Ser. Biol.* **1**, 64
[10] MIHAILOVIC, M. LJ., GRUGIC, S. A. and HADZIYEV, D. (1964) *Biochim. Biophys. Acta* **87**, 499.
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poration by polysomes from normal and opaque-2 endosperm was studied using purified *t*RNA and pH 5 enzyme fraction.

The effect of different co-factors in the incubation medium is shown in Table 4. Omitting ATP and GTP from the complete system reduced the incorporation by 88%. The incorporation was further reduced when creatine phosphate and creatine phosphokinase were omitted. Therefore, the incorporation is energy dependent. The polysomal amino acid

TABLE 4. CHARACTERISTICS OF CELL-FREE PROTEIN SYNTHESIZING PREPARATIONS FROM OPAQUE-2 ENDOSPERMS AT 15 DAYS POST-POLLINATION

Incubation mixture	Incorporation of radioactive amino acid as % of control	Incubation mixture	Incorporation of radioactive amino acid as % of control
Complete	100	Plus ribonuclease	4
-ATP, -GTP	12	-CPK, -CP, -ATP	8
		-Ribosomes	5

The complete reaction mixture contained in μmol: Tris buffer (pH 7·6) 50; $MgCl_2$, 10; KCl, 30; 2-mercaptoethanol, 8; ATP, 0·06; GTP, 0·15; each of nineteen amino acids (lacking either lysine or leucine which was added labelled) 0·0125; creatine phosphate, 5; creatine phosphokinase, 20 μg; *t*RNA, 75 μg; pH 5 enzyme 1 mg protein; polysomal RNA, 300 μg and 0·5 μCi[^{14}C]leucine or lysine as the case may be (spec. act. lysine–33 mCi/mmol; leucine–27·9 mCi/mmol) in a final vol. of 0·7 ml.

incorporation system was highly sensitive to the addition of pancreatic ribonuclease. Addition of 10 μg ribonuclease resulted in 96% inhibition. Similar inhibition by ribonuclease for various systems has been reported.[13,14]

TABLE 5. INCORPORATION OF LEUCINE VS. LYSINE BY THE POLYSOMES FROM NORMAL AND OPAQUE-2 MAIZE ENDOSPERM

Days after pollination	Polysome fraction from	Lysine / Leucine	Leucine / Lysine
15	N	0·58	1·72
	O	0·93	1·07
20	N	0·27	3·64
	O	0·61	1·63
25	N	0·29	3·47
	O	0·13	7·82

N–Normal; O–Opaque-2.

From the data presented in Table 5 it is apparent that the polysomes from opaque-2 endosperm incorporated relatively much higher lysine and lower leucine (as seen from leucine–lysine ratio) compared to normal maize at 15 and 20 days post-pollination. At 25 days post-pollination more leucine was incorporated by opaque-2 polysomes compared to

[13] MEHTA, S. L.; HADZIYEV, D. and ZALIK, S. (1969) *Biochim. Biophys. Acta* **195**, 515.
[14] HADZIYEV, D. and ZALIK, S. (1970) *Biochem. J.* **116**, 111.
[15] DENIC, M. (1970) *Improving plant proteins by nuclear Techniques. IAEA Symp.* p. 381.

normal maize polysomes. These results with leucine vs. lysine incorporation are slightly at variance with Denic[15] in that he found a higher lysine–leucine ratio throughout development. We, however, observed a lower ratio at 25 days post-pollination. This could be due to the fact that we have used a purified *t*RNA and pH 5 enzyme fraction. This period also coincides with relatively more synthesis of zein fraction in opaque-2.[7] However, it is interesting to note that after this stage of maturity the rate of protein accumulation in opaque-2 endosperm is very slow as compared to that in normal endosperm (Table 6). The increase in protein in the endosperm was only 7% in the former as compared to 36% in the latter after this stage.

TABLE 6. DISTRIBUTION OF PROTEIN IN ENDOSPERM IN OPAQUE-2 AND NORMAL MAIZE AT DIFFERENT STAGES OF MATURITY

Days after pollination	mg/endosperm		Days after pollination	mg/endosperm	
	Normal	Opaque-2		Normal	Opaque-2
15	5·7	6·3	25	13·7	11·8
20	7·1	9·6	38	21·2	12·7

DISCUSSION

In view of diverse metabolic changes brought about by the opaque-2 single recessive gene, it appears that the gene product could have a regulatory role whereby it affects in turn a wide range of other genes. It is clear that substantial protein synthesis does not take place in opaque-2 endosperm during later stages of maturity and since very little zein is formed prior to that stage the final result is depressed zein contents in the mature grain. No major differences were observed between the polysomes of normal and opaque-2 endosperm as regards nucleotide and protein composition. However, incorporation of leucine and lysine by the two preparations *in vitro* showed remarkable differences. Up to 20 days from pollination the rate of lysine to leucine incorporation in opaque-2 was about twice as high as in the normal endosperm. However, at later stage opaque-2 also incorporated considerably higher leucine and lower lysine. But since very little net protein synthesis occurred in opaque-2 endosperm after this stage, the changing pattern of leucine–lysine incorporation did not result in any considerable zein accumulation. Earlier[5–7] it was suggested that higher ribonuclease activity in opaque-2 was responsible for blocking protein synthesis at later stages of development. Our present observations as well as those of Denic[15] indicate that regulation of *m*RNA synthesis during early stages when the opaque-2 gene is functional may be playing an important role in depressing zein synthesis. The *m*RNA required for zein synthesis is probably not formed in opaque-2 in early stages of maturity.

EXPERIMENTAL

One of the high combining, well adapted inbred line Fla 3H 94-f-available in the maize programme and its opaque version were used for the present study. The opaque-2 version was obtained after 3 back crosses. The opaque and normal lines were grown at the IARI, New Delhi, during the monsoon season. Individual plants were self-pollinated.

The self-pollinated ears were harvested at the specified dates after pollination. The harvested ears were immediately chilled and kernels were dissected, pericarp and embryo were removed and endosperm collected. The endosperms were ground for 5 min in mortar and pestel and for 5 sec 2× in Waring blender, using buffer

I (0·4 M sucrose; 0·1 M Tris; pH 7·8; 50 mM, KCl; 10 mM $MgCl_2$; 4 mM β-mercaptoethanol) (1:2, w/v) in the presence of 0·8 mg/ml bentonite prepared according to Hadziyev *et al.*[11] The homogenate was filtered through 8 layers of cheese cloth, the filtrate centrifuged for 30 min at 30 000 *g* to remove nuclei and mitochondria. The supernatant obtained was layered over 2 ml 1 M sucrose solution in buffer I and centrifuged for 40 min at 60 000 rpm in Ti 75 rotor of Beckman L-2 75B ultracentrifuge. Upper two third portion of the supernatant was collected for preparation of pH 5 enzyme and other studies. The remaining was discarded and the polysomal pellet was suspended in buffer II (25 mM Tris, pH 7·6; 10 mM $MgCl_2$; 50 mM KCl; 4 mM β-mercaptoethanol) and was purified further according to Mehta *et al.*[8] Finally polysome pellet was suspended in buffer II and an aliquot taken immediately for sedimentation runs. The remainder was stored at −40° for further use. The polysomal preparation obtained had an absorbance maximum at 260 nm with a ratio of A_{260} nm/A_{280} nm close to 2·0 and the ratio of A_{260} nm/A_{235} nm close to 1·65. The sedimentation runs were carried out using Schlieren optics at 20° of Beckman L-2 75B ultracentrifuge. The observed sedimentation coefficients were corrected for viscosity and density of H_2O at 20°.

RNA extraction. *r*RNA was prepared according to the method of Mehta *et al.*,[8] and *t*RNA was prepared according to the method of Hadziyev and Zalik.[14]

Nucleotide analysis. Nucleotide analysis of *r*RNA was done according to the method used by Hadziyev *et al.*[11]

Preparation of aminoacyl-tRNA synthethases. Aminoacyl-*t*RNA synthetases were prepared from upper supernatant after sedimenting polysomes according to the method of Hadziyev and Zalik[14] except for purification with streptomycin sulphate.

Amino acid incorporation. Amino acid incorporation was measured in the incubation mixture by the method of Mehta *et al.*[13]

Acknowledgement—The authors are grateful to Dr. N. P. Datta, Project Director, NRL for providing facilities for the work.