MACROMOLECULAR SYNTHESIS DURING PLANT EMBRYOGENY

Cellular Rates of RNA Synthesis in Diploid and Polytene Cells in Bean Embryos

M. CLUTTER, T. BRADY, V. WALBOT, and I. SUSSEX. From the Department of Biology, Yale University, New Haven, Connecticut 06520. Dr. Brady's present address is the Department of Biological Sciences, Texas Tech University, Lubbock, Texas 79409, and Dr. Walbot's present address is the Department of Biology, Washington University, St. Louis, Missouri 63130.

Substantial changes occur in the rates of RNA synthesis during development of the embryo of *Phaseolus* at times when there are major shifts in histodifferentiation (16). However, the number of cells in the embryo is also increasing throughout development, and changes in overall rates of

nucleic acid synthesis may reflect the increase in cell number, rather than changes in the cellular rates of synthesis. In the present study we have measured DNA content and rate of RNA synthesis at several stages of embryogeny and have expressed the data as cellular rates of synthesis throughout development. Furthermore, since the *Phaseolus* embryo contains two distinct populations of cells, one diploid and the other polytene (4), we have compared rates of RNA synthesis in these two parts of the embryo throughout development to determine whether template activity of polytene chromosomes is quantitatively similar to that of diploid chromosomes, and whether changes in template activity occur during embryonic development.

MATERIALS AND METHODS

Plant Material

Plants of *Phaseolus coccineus* (scarlet runner bean) were field grown at Marsh Botanical Garden, New Haven, Conn. Pod lengths and seed lengths were measured, and with a previously prepared developmental timetable based on seed length (17), embryos were collected at desired developmental stages.

Terminology

The plant embryo consists of the suspensor and the organogenetic part, but there is no single term that conveniently identifies this latter. Therefore, we shall refer to the organogenetic part as *embryo*. The *embryo* consists of two storage organs, the cotyledons, and the main body or axis. In some experiments the axis alone was used.

Biochemical Measurements

Embryos were removed aseptically from seeds, the suspensor was separated from the *embryo*, and the two parts were incubated separately in 0.1-ml drops of sterile White's medium (18) with carrier-free [2,8-^{3}H]adenosine (50 μCi/ml; 3.22×10^{-6} M, New England Nuclear, Boston, Mass.) at 25°C in darkness for 2 h. 10 *embryos* or suspensors were used per treatment. ATP pool size and specific activity, the rate and the amount of RNA synthesized, and DNA content per *embryo* or suspensor were determined using the procedure of Emerson and Humphreys (8), and Brandhorst and Humphreys (6). A completely detailed account of the method and sample calculations as applied to sea urchins was published by Humphreys (11). A detailed account of the application of the method to *P. coccineus* embryos has also been published (16). The RNA synthesis rates used in the present analysis are based on data published therein.

Cell Counts

Cotyledons were sectioned transversely at 1 mm and the number of cells on the face of the section was counted. The 1-mm sections were then cut along their original longitudinal axis and the number of cells along this face were counted. From these figures and the number of sections per cotyledon, the number of cells in each cotyledon were determined. The axis was sectioned longitudinally in the median plane and the number of cells on the face of the section were counted. As the axis is cylindrical the total number of axis cells could be calculated from this single section. Suspensor cells were counted directly after teasing the suspensor lightly to separate cells and squashing the preparation under a cover slip. Counts of *embryo* cell numbers were made at four stages of development. Suspensor cell numbers were counted at the same four stages and at three intermediate stages.

Method for Deriving Genome Copy Number

The suspensor cell number stabilizes at 200 at an early stage of development, but the diploid number of chromosomes in each cell continue to replicate DNA until they reach polytene levels as high as 16,364 C in some cells (4). The level of polyteny varies within a suspensor at any stage of development with the highest level in a few cells at the basal end and levels as low as 4 C at the *embryo* end. To estimate template activity it was necessary to derive the average number of genome copies per cell. To do this the average content of DNA per diploid cell in the embryo was used (2.4×10^{-12} g) (4). Then, by taking the average total DNA found per suspensor and dividing it by the total number of cells, the average amount of DNA per cell was derived. Dividing this figure by the average diploid cell amount of DNA gave the average number of diploid copies per cell. Genome copy data herein are thus based on the diploid amount of DNA rather than the haploid which was not measured. These results show a reasonable consistency with Feulgen spectrophotometric measurements. For example, the suspensor contains 200 cells. 20 are in the basal region, 80 are in the intermediate portion, and 100 are in the part closest to the *embryo*. Feulgen spectrophotometric measurements at the 7.0-mm stage show that 20% of the basal cells are at the 4,096 C level of polyteny, 50% are at the 2,048 C level, and 30% are at 1,024 C. The remaining cells range down to the 4 C level (4). The average C level estimate for the suspensor is 333. Data based on extracted DNA show an average of 158 diploid copies per cell or 316 C (Table I).

RESULTS

Embryo Morphogenesis

Cleavage of the zygote results in two cells which are developmentally and cytologically distinct. The

TABLE I

Rates of Ribonucleic Acid Synthesis During Phaseolus coccineus Embryogeny per Organ, Cell and Genome Copy

Developmental event	Age	Seed length	Cell number	DNA content	Diploid genome copies/ cell	RNA/ Organ	RNA/Cell	RNA/ 2n copy	Suspensor cell RNA / Embryo cell RNA
	days	*mm*		$g \times 10^{-12}$/ *cell*		$g \times 10^{-10}$/ *2 h*	$g \times 10^{-15}/h$	$g \times 10^{-15}$/ *h*	
Embryo									
Meristem differentiation	8	6.0	66,835	2.4	1	48.3	36.2	36.2	—
Organogenesis	10	8.0	554,000	1.9	1	488.0	44.0	44.0	—
Cotyledons fill seed	15	15.0*	500,000	2.6	1	492.0	49.2	49.2	—
Predormancy	20	20.0*	500,000	2.1	1	0.5	0.05	0.05	—
Suspensor									
Polytenization	8	6.0	200	325.0	135	23.8	5,950.0	43.9	164
	9	6.5	200	370.0	154	33.4	8,350.0	54.2	—
	9.5	7.0	200	380.0	158	58.0	14,500.0	91.6	—
	10	8.0	200	750.0	312	139.0	34,750.0	111.2	790
	11	12.0	200	1,405.0	585	96.0	24,000.0	41.0	—
	15	15.0	200	1,740.0	725	52.6	13,150.0	18.1	267
Cell degeneration	20	20.0	185	1,513.0	630	5.0	2,702.7	4.3	54,054

* Axis only.

larger, proximal cell gives rise to the suspensor which is 1.5 mm long and consists of 200 ± 25 cells at its maximum development. Mitosis ceases in the suspensor by the 6th day of embryogeny but the cells continue to enlarge and to replicate chromosomal DNA by polytenization throughout development (5). In the late stages of embryogeny some of the basal suspensor cells may degenerate (Table I), and the suspensor does not persist into the postembryonic plant but is crushed by the elongating root during germination. The distal cell of the two-celled embryo produces the organogenetic part of the embryo. By the 7th day of embryogeny this consists of a globular mass of cells that are histologically identical. Organ formation begins by the 8th day with the outgrowth of two cotyledon primordia at the end opposite the suspensor. By the 10th day, meristems of the shoot and root have been initiated at opposite poles of the axis, and vascular procambium has been differentiated internally. By the 12th day leaf primordia have been initiated. Growth of the cotyledons to fill the seed cavity has been completed by the 15th day, and seed maturation and dormancy have been completed by the 50th day, at which time the *embryo* consists of 9×10^6 cells of which 5×10^5 are in the axis.

RNA Synthesis

RNA synthesis per *embryo* cell increases to its maximal rate by day 10 which is the time when organogenesis and histodifferentiation are proceeding rapidly. It is maintained at a high rate through day 15, but by day 20 when the *embryo* has reached its full size, the rate of RNA synthesis per axis cell has decreased to less than 1.0% of the highest value (Table I). The rate of RNA synthesis per suspensor cell is higher than that in *embryo* cells at all stages of development examined (Table I). The rate rises to a maximum on the 10th day and then declines during later stages of development. Suspensor cellular rates of RNA synthesis were compared directly with *embryo* rates at four stages of development for which the average number of cells were counted. The suspensor cell rate was always hundreds of times greater than the *embryo* rate (Table I).

Template Activity of Diploid and Polytene Chromosomes

The amount of RNA synthesized per hour expressed per unit of DNA permits a comparison to be made between the template activity of polytene chromosomes in the suspensor cells and

that of diploid chromosomes in the *embryo* cells (Table I). In the *embryo*, template activity measured as femtograms of RNA synthesized per hour per cell (diploid copy) increases throughout embryogeny until the final predormancy state when it approaches zero. In the suspensor, template activity is approximately the same as that in diploid cells during early development. It rises to a maximum at day 10 which is more than twice the maximal rate in *embryo* cells, then declines during the later stages of suspensor development.

DISCUSSION

From the time of the asymmetric division of the zygote there are two developmentally distinct cell populations in the embryo of *Phaseolus*. The organogenetic part (called *embryo* here) consists of a population of diploid cells that is enlarging through continued mitosis, and is diversifying through histodifferentiation and organogenesis. The suspensor is a smaller cell population which is numerically stabilized through most of development, but in which DNA synthesis is continuing by chromosomal polytenization. Thus cell differentiation in these two populations proceeds under very different conditions of nuclear activity.

At all times during development, RNA synthesis in the two parts of the *embryo* is strikingly different. At the organ level, the embryo is always more active except for the final predormancy stage. However, on a cellular basis, the rate of RNA synthesis in the suspensor cells is always more than 100 times greater than that in diploid *embryo* cells. The polytene chromosomes of *Phaseolus* thus appear to be biochemically active. That this is true is supported by other evidence: cytological observation of these chromosomes revealed that they are largely euchromatic (5); and autoradiographs of incorporation experiments showed that [^{3}H]uridine is incorporated in all portions of the chromosomes except in the centromeric heterochromatin at different times during development (3), which suggests that large areas may be transcribing RNA.

The interrelations between DNA and RNA synthesis in the suspensor cells are interesting. The DNA content of a suspensor cell increases throughout development. Since the number of gene copies presumably increases with polytenization, the rate of RNA synthesized by suspensor cells was compared at several developmental stages with the RNA synthesis rate of *embryo* cells in the present studies to see whether there might be a gene dosage effect. By the 8th day of development, suspensor cells were 164 times more active than *embryo* cells. The number of diploid copies of the genome present per suspensor cell was 135 (Table I). Thus, RNA synthesis appears to be proportional to the genome copy number. However, by 10 days the suspensor cells were 790 times more active than the *embryo* cells, while the average number of diploid genome copies per suspensor cell was 312. The polytene cells at this stage of development were more than twice as active as would be predicted by the number of genome copies. Also, later in development RNA synthesis was at variance with expected results. By day 20, suspensor cells were 54,000 times as active as *embryo* cells, while the average number of genome copies per cell was 630; but this large difference can be accounted for by the very low rate of RNA synthesis in the *embryo* where it is known that RNA synthesis declines before dormancy of the seed (15). In the suspensor, too, RNA synthesis was declining, but not as rapidly.

It is necessary to explain why suspensor template activity is higher than would be predicted by the number of genome copies during mid-development. There may be localized gene amplification, as has been observed in *Rhynchosciara* polytene chromosomes (7), but current evidence suggests otherwise. First, saturation rRNA-DNA molecular hybridization experiments have shown no evidence of amplification of the ribosomal cistrons in *Phaseolus*[1]; moreover, Feulgen spectrophotometric measurements of DNA have shown that during polytenization there is an exact doubling with each round of replication from 2 C to 16,364 C (4). Therefore, other explanations must be explored.

In *Xenopus laevis* large increases in rRNA synthesis during neurulation cannot be accounted for by gene amplification because it is known that the ribosomal cistrons in somatic cells remain at a constant level throughout development (9). The explanation generally given for this increase is that during development there is differential template activity, that all the available cistrons are not used at all times of transcription, and that in this case either a larger number of cistrons are being utilized or there is a greater overall rate of transcription of the same number of cistrons. In the suspensor the same explanation could be applied.

Additional evidence which shows that it is not necessary to invoke gene amplification to explain a

[1] A. Lima De Faria, personal communication.

high rate of RNA synthesis in specialized cells is the case of the silk fibroin message synthesized in the polyploid silk gland cells (between 10^5 and 10^6 C) of *Bombyx mori* (14). No amplification occurred, but polyploidy undoubtedly played a role in the synthesis of vast amounts (about 300 μg/cell) of the protein (14). Cellular RNA synthesis data from *Bombyx* as a function of copies of the genome have not been published.

Because the rate of RNA synthesis in the polytene cells of *Phaseolus* is hundreds of times greater than that in diploid cells, it seems clear that there is some advantage to replication of the genome in the absence of cytokinesis. Indeed, there is evidence (both direct and circumstantial) that this phenomenon is not limited to beans but is used in other organisms where large amounts of RNA or special products are synthesized in differentiated cells. The most complete data available are from the silk gland cells (14), but other examples less well elucidated are insect nurse cells (→4,000 C) which provide RNA for the developing oocyte (2), insect footpad cells (→4,096 C) which secrete cuticle (19), mammalian embryo trophoblast cells (→1,024 C) which are actively engaged in enzyme synthesis (20, 13), and dipteran salivary gland cells (→16,000 C), where strong evidence supports a direct relationship between a protein component in the gland secretion and puffing activity of the chromosomes, (1, 10, 12).

The accumulating evidence indicates that polyteny and postmitotic polyploidization have developmental significance. It must be noted, however, that these are mechanisms employed in terminally differentiating organs or cells for mass transcriptional events, and should not be considered as either the cause or the consequence of differentiation in general.

SUMMARY

The embryo of *Phaseolus coccineus* consists of two cell populations, one diploid, the other polytene. Cellular rates of RNA synthesis were measured at different times during development in embryos removed from the seed and incubated in medium containing [^{3}H]adenosine. RNA synthesis in both diploid and polytene cells reached a maximum at mid-development and then declined, but the rate of RNA synthesis per polytene cell was always hundreds of times greater than the rate in diploid cells. The rate of RNA synthesis per unit of DNA in diploid embryo cells remained fairly uniform at all stages measured. In the polytene cells of the suspensor, the rate of RNA synthesis per diploid copy of DNA was initially similar to the rate in diploid cells, but increased at mid-development to more than twice as much, and then declined. It is postulated that polyteny provides a mechanism by which a tissue can rapidly produce large amounts of RNA without committing energy resources to processes associated with new cell formation.

This work was supported by National Science Foundation grant GB 8709 to M. Clutter and I. Sussex. V. Walbot was supported by a National Science Foundation predoctoral fellowship, and T. Brady by United States Public Health Service training grant HD-00032.

Received for publication 8 November 1973, and in revised form 19 August 1974.

REFERENCES

1. BEERMANN, W. 1961. Ein Balbiani-Ring als Locus einer Speicheldrüsenmutation. *Chromosoma (Berl.).* **12**:1–25.
2. BIER, K., W. KUNZ, and D. RIBBERT. 1967. Struktur und Funktion der Oocyten Chromosomen und Nukleolen sowie der Extra-DNS während der Oogenese panoistischer und meroistischer Insekten. *Chromosoma (Berl.).* **23**:214–254.
3. BRADY, T. 1970. Activities of polytene chromosomes in *Phaseolus*. *J. Cell Biol.* **47**(2, Pt. 2):23 *a* (Abstr.).
4. BRADY, T. 1973. Feulgen cytophotometric determination of the DNA content of the embryo proper and suspensor cells of *Phaseolus coccineus*. *Cell Diff.* **2**:65–75.
5. BRADY, T., and M. CLUTTER. 1974. Structure and replication of *Phaseolus* polytene chromosomes. *Chromosoma (Berl.).* **45**:63–79.
6. BRANDHORST, B. P., and T. HUMPHREYS. 1971. Synthesis and decay rates of major classes of deoxyribonucleic acid-like ribonucleic acid in sea urchin embryos. *Biochemistry.* **10**:877–880.
7. BREUER, M. E., and C. PAVAN. 1955. Behavior of polytene chromosomes of *Rhynchosciara angelae* at different stages of larval development. *Chromosoma (Berl.).* **7**:371–386.
8. EMERSON, C. P., and T. HUMPHREYS. 1971. A simple and sensitive method for quantitative measurement of cellular RNA synthesis. *Anal. Biochem.* **40**:254–266.
9. GURDON, J. B. 1967. Control of gene activity during the early development of *Xenopus laevis*. *In* Heritage from Mendel. R. A. Brink and A. D. Styles, editors. University of Wisconsin Press, Madison, Wis. 203.
10. GROSSBACK, U. 1969. Chromosomen-Aktwität und biochemische Zelldifferenzierung in den Speicheldrüsen von *Camptochironomus*. *Chromosoma (Berl.).* **28**:136–187.

11. HUMPHREYS, T. 1973. Quantitative measurement of RNA synthesis. *In* Molecular Techniques and Approaches in Developmental Biology. M. Chrispeels, editor. John Wiley & Sons, Inc., New York. 141–163.
12. RASCH, E. M. 1970. DNA cytophotometry of salivary gland nuclei and other tissue systems in Dipteran larvae. *In* Introduction to Quantitative Cytochemistry II. G. L. Wied and G. F. Bahr, editors. Academic Press, Inc., New York. 357–397.
13. SHERMAN, M. I. 1974. The relationships between the early mouse embryo and its environment. *In* The Developmental Biology of Reproduction. C. L. Markert, editor. 33rd Symposium of the Society for Developmental Biology. Academic Press, Inc., New York. In press.
14. SUZUKI, Y., L. P. GAGE, and D. BROWN. 1972. The genes for silk fibroin in *Bombyx mori*. *J. Mol. Biol.* **70**:637–649.
15. WALBOT, V. 1971. RNA metabolism during embryo development and germination of *Phaseolus vulgaris*. *Dev. Biol.* **26**:369–379.
16. WALBOT, V., T. BRADY, M. CLUTTER, and I. SUSSEX. 1972. Macromolecular synthesis during plant embryogeny: rates of RNA synthesis in *Phaseolus coccineus* embryos and suspensors. *Dev. Biol.* **29**:104–111.
17. WALBOT, V., M. CLUTTER, and I. SUSSEX. 1972. Reproductive development and embryogeny in *Phaseolus*. *Phytomorphology*. **22**:59–68.
18. WHITE, P. R. 1943. A Handbook of Plant Tissue Culture. Jacques Cattell Press, Tempe, Ariz.
19. WHITTEN, J. M. 1969. Coordinated development in the fly foot: sequential cuticle secretion. *J. Morphol.* **127**:73–104.
20. ZYBINA, E. V. 1970. Features of polyploidization patterns of trophoblast cells. *Tsitologiya*. **12**: 1081–1095.