Changes in Nuclear Protein Contents of Root Cells during Germination

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Changes in the protein composition of nuclei occur in many cell types as they undergo transitions from one state to another. Total histone content of nuclei shows only a 2-fold range of values over a cell cycle (Prescott 1966, Borun 1975) but they undergo modifications in the degree of acetylation or phosphorylation that have been related to chromatin activation (Chahal, Matthews and Bradbury 1980) or chromosome condensation (Bradbury, Inglis, Matthews and Langan 1974, Matsumoto, Yasuda, Mita, Marunouchi and Yamada 1980). The onset of differentiation may be accompanied by a decrease in total nuclear protein content (Mirsky and Ris 1949, Le Stourgeon, Rusch, Totten and Forer 1974). Transition from the G_0 state to the proliferative state includes increases in the number of different types of proteins (Stein and Baserga 1970, Le Stourgeon and Rusch 1971, Levy, Levy, Rosenberg and Simpson 1973, Jeter and Cameron 1974) and in the total acidic protein content of nuclei (Mirsky and Ris 1949, Le Stourgeon *et al.*, 1974). This latter type of transition parallels the change in meristem cells of roots of germinating seeds: cells of the dormant seed become activated and enter a proliferative phase as the seed germinates.

In the radicle of the dry seed of *Vicia faba* about 90% of the cells have a G_1 DNA content (Jakob and Bovey 1969). When seeds imbibe water, cells become activated; the first cells to complete a cell cycle enter mitosis in a semi-synchronous wave (Davidson 1966, Murin 1967). In the subsequent 2-3 cycles: 1) the root meristem achieves a steady-state condition; as it does so, mean cell area decreases but mean nuclear area remains fairly constant (Thomas and Davidson 1981, 1982, 1983). Since, in other systems, an increase in nuclear protein content is associated with a shift from the G_0 to the proliferative condition, we have measured nuclear protein contents during the first 144 hours of germination in order to determine: 1) whether mean protein content of nuclei changes as root meristem cells progress from the dormant condition into a proliferative stage in spite of the constancy of mean nuclear area; 2) what is the relationship between mean area and mean protein content?

Mean protein content of nuclei, as we report here, decreased as cells became proliferatively active, suggesting that the relationship between protein and size of nuclei is somewhat flexible. In order to explore this possibility we treated roots with 5-aminouracil: this extends the duration of G_2 (Socher and Davidson 1971) and the cells and nuclei delayed in their progress through interphase show significant increases in size (Davidson, Golding and Armstrong 1978). Mean nuclear pro-

tein contents were determined. Though some increase in protein content was found, it did not equal the nuclear size increase in magnitude. This supports the conclusion that, during the first few days of germination, the relationship between size and protein content of nuclei is not fixed. These results agree with those from other systems that variation in dry mass (Lyndon 1967) and protein content (Armstrong and Davidson 1982) is to some extent independent in variation of size of nuclei.

Materials and methods

Beans, *Vicia faba* L. cv. Sutton's Prolific Longpod, were notched above the embryo axis, placed on their sides between two layers of cheese cloth and distilled water was added to a depth of 5–10 mm. After 24 hours the seed coats were removed, the beans rinsed in distilled water and growing roots were obtained in the following ways: i. seeds were skewered with stainless steel rods and placed, with the root down, in aerated distilled water, which was changed every 24 hours. They were fixed, at intervals, upto 144 hours from the start of imbibition; ii. seeds were planted in moistened sand (25 ml of water/100 ml of sand). After 96 hours from the beginning of imbibition, seedlings were removed from the sand, rinsed, skewered with steel rods and grown in tanks of aerated distilled water.

For 5-aminouracil (5-AU) treatments, some seedlings grown in moistened sand and then transferred to tanks of water, as described above, were placed in tanks containing a 4 mM solution of 5-AU. They were treated for 6–24 hours. Seeds were imbibed and all seedlings were grown at 20°C.

Protein Determinations. Nuclei were extracted from the 1–2 mm of the apex of the root meristem using a method modified from Van't Hof (1975). At each time 5 root tips were placed in ice-cold 4% formaldehyde (v/v) in 0.132 M phosphate buffer (pH 6.8). After 5 min they were transferred to ice-cold 0.132 M phosphate buffer. In order to isolate nuclei, each meristem was placed in 2 drops of fixative on an ice-cold alcohol cleaned slide and macerated. This released the nuclei from the cells. Large pieces of cellular debris were removed and the slides were partially flooded with fixative. Nuclei were spread around the slide in order to minimize cytoplasmic background and then left to air dry.

Proteins were stained using a modification of the method of Mitchell (1967). In order to make comparisons between different samples valid, all slides were stained at the same time. Slides were placed in 1:1 mixture (v/v) of acetone: ethanol for 30 min and then in 0.15 ml 2:4 dinitro-1-fluorobenzene (DNFB) in 100 ml ethanol plus 1 ml 1 M sodium bicarbonate and 5 ml distilled water. Nuclei were stained for 12 hours at 65°C. After staining, slides were washed in 70% ethanol (15 min), warm distilled water (30°C, 15 min) and then dehydrated in 70% ethanol (5 min), 95% ethanol (10 min), 100% ethanol (2 changes for 10 min each) and xylene (5 min). Nuclei were mounted in Permount.

Measurements of total nuclear protein were made using the 2 wavelength method of Patau (1952). The wavelengths used were 415 and 445 nm; i.e. the peak and 50% of the peak absorption for the DNFB-protein complex. All measure-

ments were made, at a magnification of $630\times$, using a Leitz microspectrophotometer ($NA=0.85$). At each fixation time, 20 to 144 hours from the beginning of imbibition, 100 interphase nuclei, 20 from each of 5 meristems, were measured. Length and width of each nucleus were measured with an ocular micrometer in order to calculate nuclear area.

Results

Protein content of nuclei was determined 20 to 144 hours from the onset of germination. Mean nuclear protein contents were similar, in roots of the same age from the onset of germination, whether they had been grown in water or in moistened sand; e.g. 68 ± 23 and 66 ± 20 A.U. at 50 hours (Tables 1 and 2). The pattern of change in mean protein contents was also similar in the two groups of roots; it increased upto 50 hours, as seeds moved through S and into G_2 of the first cell cycle, and subsequently decreased. In roots grown in water, mean nuclear protein con-

Table 1. Mean $\pm$ S.D. and range in area and in protein contents of nuclei of primary roots of *Vicia faba*. Each value is based on 100 nuclei. Seeds were imbibed for 24 hours and then grown in tanks of aerated distilled water for upto 144 hours

Hours after addition of water	Nuclear area (μm^2)		Nuclear protein (A.U.)	
	mean $\pm$ S.D.	range	mean $\pm$ S.D.	range
20	97 ± 28	60–172	55 ± 18	32–112
36	97 ± 27	52–156	62 ± 25	34–150
50	101 ± 24	62–177	68 ± 23	30–158
68	102 ± 24	62–169	50 ± 17	19– 89
96	96 ± 18	62–145	43 ± 14	19– 86
144	89 ± 21	52–171	38 ± 11	18– 61

tent fell by 44% between 50 and 144 hours (Table 1) and by 33%, between 50 and 96 hours, in roots grown in moistened sand (Table 2). In both sets of roots the decreases are significant ($P=0.05$); i.e. 68 ± 23 to 38 ± 11 A.U. and 66 ± 20 to 44 ± 13 A.U. (Tables 1 and 2). The modal value of nuclear protein content also increased upto 50 hours and then decreased (Fig. 1).

The range of values of nuclear protein contents showed a similar pattern of change. It increased upto 50 hours and subsequently decreased. We conclude that nuclear protein contents decrease when root meristematic cells of germinating beans become active in proliferation. This decrease in mean values and in the range of values occurred without a decrease of similar magnitude in nuclear size: i.e. a 44% decrease in mean protein contents between 50 and 144 hours was accompanied by only an 11.8% decrease in mean nuclear area (Table 1). Thus the average concentration of total protein, per unit volume of nucleus, decreases significantly as cells of the root meristem achieve an asynchronous, steady-state condition. This decrease has been analysed in greater detail by selecting nuclei of specific sizes and comparing their protein contents.

Protein Contents of Nuclei of Selected Area Classes

In order to determine whether the pattern of changes in mean nuclear protein content occurred in nuclei of specific area classes we selected nuclei of areas 71, 82,

Table 2. Mean $\pm$ S.D. area and mean $\pm$ S.D. and range of protein contents of nuclei. Seeds were imbibed in 5 mm of water for 24 hours, grown in moistened sand (25 ml water/100 ml sand) from 24 to 96 hours and then transferred to tanks of water. Some seedlings were transferred, after 24 hours in water, to a 4 mM solution of 5-AU for 6, 14 or 24 hours. Each value is based on 100 nuclei

Hours in			Nuclear area (μm^2)	Nuclear protein content (A.U.)	
sand	water	5-AU	mean $\pm$ S.D.	mean $\pm$ S.D.	range
36	—	—	94 $\pm$ 26	60 $\pm$ 21	33–113
50	—	—	104 $\pm$ 22	66 $\pm$ 20	24–116
68	—	—	96 $\pm$ 17	46 $\pm$ 13	19– 77
96	—	—	93 $\pm$ 21	44 $\pm$ 13	15– 76
96	30	—	89 $\pm$ 19	42 $\pm$ 16	20– 89
96	48	—	93 $\pm$ 29	41 $\pm$ 15	16– 84
96	24	6	98 $\pm$ 24	38 $\pm$ 14	19– 87
96	24	14	101 $\pm$ 30	47 $\pm$ 15	17– 84
96	24	24	106 $\pm$ 28	43 $\pm$ 15	12– 87

Fig. 1. Percent frequency histograms of nuclear protein contents in root meristems of *Vicia faba*. Seeds were imbibed in 5–10 mm of water for 24 hours and then grown in tanks of water. The hours given are timed from the start of imbibition. At each time, 100 nuclei were isolated from 5 root meristems. Protein values are given in arbitrary units (A.U.).

93 and 100 μm^2 . They were taken from those nuclei used for Table 1 and they were chosen because they yielded, in most cases, samples of 12–20 nuclei for each area chosen. These selected nuclei, chosen solely on the basis of their area, demonstrate that the conclusions presented earlier for changes in mean protein content of an overall sample of 100 nuclei, are borne out by nuclei of specific area classes. That is, for each area class mean protein content increases upto 50 hours and decreases from 50 to 144 hours (Table 3). The additional conclusion to be drawn is that small nuclei at 20–50 hours have a higher mean protein content than larger nuclei at 144 hours; cf. the 71 μm^2 class at 20 hours, with a mean protein content of 44.9 ± 9.8 A.U., with the 110 μm^2 area class at 144 hours, with a mean protein content of 41.7 ± 10.4 A.U.

Nuclear protein contents cover a 2- to 3-fold range of values for a specific nuclear size class (Table 3). The extent of the overlap in protein content of nuclei

of identical areas is demonstrated by comparing probit plots of protein content of three samples of nuclei. Nuclei of areas 60, 82 and $100\ \mu\text{m}^2$ were selected from the total sample scored (Table 4); they were chosen because their sample sizes were large, 83, 251 and 181 nuclei respectively, and because the 60– $100\ \mu\text{m}^2$ spread covers almost a 2-fold increase in nuclear area. The probit plots (Fig. 2) reveal the considerable overlap in protein contents of nuclei of different areas: $\sim 65\%$ of the $100\ \mu\text{m}^2$ area nuclei had protein values that fell in the range covered by the $60\ \mu\text{m}^2$ area nuclei, i.e. 18–65 A.U. (Fig. 2).

Table 3. Mean $\pm$ S.D. and range of proteins in nuclei of selected area classes. Number of nuclei in each area class is given. Data taken from the nuclei reported in Table 1

Hours from addition of water	Nuclear area (μm^2)	Number of nuclei	Nuclear protein (A.U.)	
			mean $\pm$ S.D.	range
20	71	17	44.9 $\pm$ 9.8	31.5–69.4
	82	16	50.0 $\pm$ 7.7	38.4–66.2
	93	12	50.4 $\pm$ 13.7	40.8–91.1
	110	12	65.1 $\pm$ 25.6	34.0–112.3
36	71	16	52.4 $\pm$ 13.6	33.6–82.2
	82	21	52.4 $\pm$ 10.9	37.4–78.4
	93	13	56.9 $\pm$ 14.2	37.4–80.8
	110	13	72.0 $\pm$ 21.0	49.1–114.2
50	71	16	48.4 $\pm$ 15.0	30.0–82.3
	82	15	64.4 $\pm$ 14.0	43.5–89.0
	93	19	70.0 $\pm$ 12.7	43.2–92.3
	110	19	76.6 $\pm$ 21.5	30.6–115.9
68	71	7	35.5 $\pm$ 10.3	22.0–49.1
	82	27	47.6 $\pm$ 13.4	22.0–69.0
	93	13	54.7 $\pm$ 14.5	39.4–78.2
	110	15	51.8 $\pm$ 16.0	24.9–86.1
96	71	12	29.6 $\pm$ 5.6	23.3–41.2
	82	25	36.8 $\pm$ 11.4	18.7–63.5
	93	18	50.0 $\pm$ 12.6	23.7–72.2
	110	20	52.0 $\pm$ 10.5	22.0–68.0
144	71	24	33.5 $\pm$ 9.2	22.1–54.8
	82	29	38.5 $\pm$ 9.3	24.4–58.9
	93	16	43.3 $\pm$ 7.6	28.7–60.7
	110	8	41.7 $\pm$ 10.4	27.9–54.0

The probit plots also revealed that protein contents of nuclei were not normally distributed (Fig. 2) since none of the three distributions appeared to fit a single straight line. From the range in protein contents of nuclei (Table 3; Fig. 2) it appears that, for individual nuclei, area may vary, to a large extent, independently of the amount of protein present. When mean protein content is plotted against mean nuclear area, however, a clear relationship between these two parameters emerges.

Nuclei were grouped into size classes (Table 4). Some classes contained few nuclei, i.e. 1–11, and their mean protein contents showed little correlation between

nuclear area and protein content. Therefore we plotted mean protein content against nuclear area for samples of 40 or more nuclei and we added, at the two extremes of the range of nuclear areas a sample of 20 nuclei, the $49 \mu\text{m}^2$ class, and one of 17 nuclei, the $154 \mu\text{m}^2$ class (Table 4).

Fig. 2. Percent cumulative frequency plot (Probit) of protein contents of nuclei of area $60 \mu\text{m}^2$ (●), $82 \mu\text{m}^2$ (□) and $110 \mu\text{m}^2$ (▲). The three samples consisted of 83, 251 and 181 nuclei, respectively. Protein values are given in arbitrary units.

Fig. 3. Mean nuclear protein content plotted against nuclear area. Nuclear areas were grouped in $5.5 \mu\text{m}^2$ intervals. Each mean nuclear protein content is based on a sample of 40 or more nuclei, except the values of $49 \mu\text{m}^2$ and $154 \mu\text{m}^2$, which are based on 20 and 17 nuclei. Protein contents are given in arbitrary units (A.U.).

tment with 5-AU brings mean nuclear area back to the value seen at 50 hours, i.e. $106 \pm 28 \mu\text{m}^2$, cf. $104 \pm 22 \mu\text{m}^2$, mean protein content did not return to the initial high value: it was only 43 ± 15 A.U. at $96 \pm 24 + 24$ hours, cf. 68 ± 20 A.U. at 50 hours

The plot (Fig. 3) reveals a clear linear relationship between the two parameters. The line has a slope of 0.474 and a y-intercept of 6.45 A.U. Thus, when fairly large samples of nuclei are scored their mean protein content increases in a regular fashion as their mean area increases. The correlation coefficient for the samples plotted in Fig. 3 was calculated: $r=0.993$. By extrapolation it can be seen that protein content of a nucleus increased—1.8 to 1.9-fold for one doubling in nuclear area.

Response to Treatment with 5-AU

Seedlings grown in sand from 24 to 96 hours were transferred to tanks of water and after 24 hours they were treated with 5-AU for 6, 14 or 24 hours. Treatment with 5-AU produced a 14% increase in mean nuclear area: from $93 \pm 29 \mu\text{m}^2$ in roots grown in water for 48 hours to $106 \pm 28 \mu\text{m}^2$ in roots treated with 5-AU for 24 hours (Table 2). But there was only a minimal increase in mean protein content, i.e. 41 ± 15 A.U. to 43 ± 15 A.U. (Table 2). The range of nuclear protein contents also changed very little over the 24 hour treatment with 5-AU (Table 2). Although treat-

(Table 2). This is further evidence that, at different stages of growth, mean protein content may vary independently of mean nuclear area.

Discussion

The first wave of semi-synchronous mitosis appears in root meristems of germinating *V. faba* at about 50 hours (Thomas and Davidson 1981). From the

Table 4. Mean $\pm$ S.D. and range of protein contents in root nuclei.

Seedlings were grown in water or in moistened sand (25 ml of water/100 ml sand) for up to 144 hours from the onset of imbibition. The 1202 nuclei scored were divided into area size classes; the class interval was $5.5 \mu\text{m}^2$

Nuclear area (μm^2)	Number of nuclei	Nuclear protein (A.U.)		
		mean	S.D.	range
49	20	30 $\pm$ 9		15– 48
55	1	34		
60	83	37 $\pm$ 10		18– 65
66	2	43 $\pm$ 11		36– 51
71	171	42 $\pm$ 14		22– 82
77	21	37 $\pm$ 11		19– 54
82	251	47 $\pm$ 15		19– 98
88	25	41 $\pm$ 12		22– 71
93	196	55 $\pm$ 17		22– 110
99	24	45 $\pm$ 9		26– 59
104	27	51 $\pm$ 16		31– 92
110	181	58 $\pm$ 21		22– 136
115	8	44 $\pm$ 13		45– 55
121	90	66 $\pm$ 23		23– 132
126	11	50 $\pm$ 16		24– 84
132	5	60 $\pm$ 13		42– 77
137	43	77 $\pm$ 26		39– 150
143	4	57 $\pm$ 20		40– 80
148	9	71 $\pm$ 28		42– 128
154	17	79 $\pm$ 25		45– 130
165	3	53 $\pm$ 16		36– 68
170	7	85 $\pm$ 38		48– 158
176	2	68 $\pm$ 22		53– 84
181	1	40		

onset of imbibition to 50 hours, mean cell and nuclear size increase (Thomas and Davidson 1983) and mean nuclear protein content increases (Tables 1 and 2). These changes parallel those seen in animal cells in the transition from G_0 to a state of active cell growth (Le Stourgeon *et al.* 1974).

Once cells have completed their first mitosis and begin progress through the second and subsequent cell cycles, mean protein content of nuclei decreased. Though the decrease was progressive from 50 to 144 hours (Table 1) it appears to occur in two stages. Between 50 and 96 hours root meristems undergo a number of changes: 1)

mean cell cycle duration decreases, to ~ 20 hours (Thomas and Davidson 1982); 2) mean cell area decreases (Thomas and Davidson, 1983); 3) mean nuclear protein content decreases (Tables 1 and 2). But from 96 to 144 hours mean area and mean protein content of nuclei show only slight changes (Tables 1 and 2). In this period, root meristems gradually take on the characteristics of a steady state system and the growth pattern of their cells is similar to that of other cells (Prescott 1955, Zetterberg and Killander 1965).

The analysis of the changes in cell, nuclear and protein parameters during the germination of *V. faba* suggests that the rates of i) cell growth and ii) progress through interphase are not closely coordinated. The result is that cell cycle duration is not long enough to allow cells to double their size or their nuclear protein contents, though they do double their nuclear area. This means that nuclei are competent to complete a replication and division cycle in spite of reduction in their protein content. Though some nuclear proteins must be essential for maintaining chromatin structure and gene activity, others can be lost, or reduced in amount, without affecting the ability of the nucleus to replicate and divide.

Protein content of nuclei also decreases after treatment with caffeine and, as with the results reported here, affected cells remain able to complete a cell cycle and divide (Armstrong and Davidson 1982). The difference between the two systems in which a reduction in mean nuclear protein content occurs is significant. Here we describe the reduction that occurs between 50 and 96 hours of germination: the proteins lost could either be ones required to maintain the quiescent condition of nuclei while the seeds were dormant or ones that must be lost in order to allow nuclei to undergo the reprogramming necessary for them to give rise to a steady-state population of proliferating cells. Similar proposals of programming for cell cycle changes have been made for bacteria (Kubitschek 1966) and animal cells (Liskay, Leonard and Prescott, 1979). With caffeine treated roots, however, the drop in protein content took place in nuclei of cells that were already in a steady-state condition. Even in these nuclei, therefore, a proportion of their protein molecules may be lost without reducing their ability to replicate or divide (Armstrong and Davidson 1982). The fraction of nuclear proteins that appears to be dispensable may be similar to those identified in *A. proteus* as nonstructural proteins that move freely between cytoplasm and nucleus (Goldstein and Ko 1981). The distribution of such proteins between nucleus and cytoplasm may be determined by physiological state of a cell, or by treatment, e.g. with caffeine or 5-AU. Changes in the relative amounts of such proteins in nuclei at different stages of root growth or in different cell lineages within a meristem may help to explain both the variation 1) in protein content of nuclei of a specific size class (Table 4) and 2) in nuclear dry mass at a specific stage of interphase (Lyndon 1967). The question that remains, however, concerns the mechanism that enables nuclei to vary so markedly in size and in protein content.

Summary

Total protein content and area of nuclei were determined for cells of the primary

root of *Vicia faba* during the first 144 hours following the onset of imbibition. We wished to determine what change, if any, occurs in protein contents of nuclei as meristematic cells move from a dormant into a proliferative condition. Nuclear proteins were stained with dinitrofluorobenzene and protein content, in arbitrary units, was determined by microspectrophotometry. In the first 50 hours mean protein content of nuclei increase; this, as we have shown, is the period in which cells and nuclei increase in size as they complete their first cell cycle. During subsequent cell cycles, cell area decreases markedly, nuclear area decreases to a lesser extent; accompanying these decreases, the mean and the range of protein contents of nuclei decreased significantly. Thus, as the proliferating population of cells of a root meristem becomes steady-state, there is an absolute decrease in the concentration of total proteins within the nucleus. This was revealed particularly clearly by comparing nuclei of identical areas from roots of different ages. Increases in nuclear size were induced by treatment with 5-aminouracil for upto 24 hours: this did not result in a significant increase in protein content of nuclei. These results agree with those from untreated roots that area and protein content of nuclei can, to a considerable extent, vary independently of one another.

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