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Repression of genetic activity in root meristem cells by peptidic factor derived from male sex organs of *Chara*

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Abstract

Present autoradiographic investigations using ^{3}H-uridine and ^{3}H-leucine incorporation indicate that preincubation of primary roots of *Melandrium noctiflorum* with antheridial chromatin condensation factor (ACCF), a low molecular mass peptidic factor (4.5 kDa) obtained from maturing male sex organs of *Chara tomentosa*, leads to a marked reduction of transcription and translation in G1, G2 and endopolyploid cells (8C DNA level). Moreover, silver staining of cells reveals considerable decrease in number and sizes of nucleoli. The similarities that exist between the "innate" processes within developing antheridial filaments of *Chara* and the genetic repression induced by antheridial peptidic extracts in root cells of *M. noctiflorum* seems to provide further positive evidence for the presence of a highly specific factor that contributes to nuclear re-patterning of differentiating germ line cells in male sex organs of *Chara*.

Additional key words: spermatogenesis, cell cycle, nucleoli, transcription.

Introduction

Haploid germ line cells which form antheridial filaments (a.f.) in male sex organs of *Chara* offer a unique system where different modes of "negative" transcriptional control are encountered, each representing distinct functional context and a relevant set of regulatory agents. The "proliferative period" of spermatogenesis, comprising a series of synchronous cell cycles multiplies spermatids providing for an exponential sequence of developmental stages with the filaments composed of 1, 2, 4, 8, 16, and

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Abbreviations: ACCF - antheridial chromatin condensation factor; a.f. - antheridial filaments; C-value - DNA content per genome; NOR - nucleolus organizing region; PHA - phytohemagglutinin; THB - Tris-HCl buffer, pH 7.5.

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32 or 64 cells. Concurrently, the interphase periods needed to proceed throughout every stage and the maximum cell dimensions attained at mitosis become considerably shortened (Godlewski and Olszewska 1973, Maszewski and Kołodziejczyk 1991).

A great number of cellular processes which accompany progression of a.f. throughout the proliferative period of spermatogenesis implicate that the gradual changes of their functional activities correlate closely with the increasing proportion of condensed chromatin (*e.g.* Olszewska 1978, Maszewski and Kołodziejczyk 1991, Maszewski and Kaźmierczak 1995). Morphogenetic transformations which specify successive generations of spermatids manifest by a considerable reduction of nuclear (Olszewska 1974a) and nucleolar profile areas (Kwiatkowska and Maszewski 1985, Maszewski 1994) and an apparent increase in size and number of chromocentres (Olszewska 1978). The achieved level of chromatin condensation at consecutive late G2 phases contributes directly to the extent of chromosomes shortening at each of the forthcoming M-phases (Olszewska 1978, Maszewski 1997). Accordingly, alterations of nuclear architecture correspond with the gradual repression of transcription (Olszewska 1974b, Kwiatkowska and Maszewski 1979, Maszewski and Kwiatkowska 1984) and protein synthesis (Olszewska and Godlewski 1972, Maszewski 1981). Structural and functional changes proceed in this way until the cells converge upon a specific point that turns off their proliferation and engage a new pool of developmental mechanisms qualified to arrange differentiation of spermatozoids and terminal inactivation of their nuclei, facilitated by exchange of somatic histones into protamine-like proteins (Robert 1984).

Recent studies with crude and peptidic extracts obtained from maturing antheridia of *Chara* (with spermatids terminating their proliferative period of development) have demonstrated that factors engaged in morphogenetic transformations of a.f. are capable to induce significant structural and functional effects in as diverse systems as root meristems (Maszewski 1997, Kaźmierczak and Maszewski 1997), fern gametophytes, human PHA-stimulated lymphocytes (Maszewski 1995), HeLa cells and fission yeasts (*Schizosaccharomyces pombe*; Maszewski *et al.*, unpublished). Biological assays indicated that the activity eliciting increased condensation of chromosomes and/or altered physiological response of cells is confined merely to a fraction of low molecular mass (4.5 kDa) peptides, further termed as antheridial chromatin condensation factor (ACCF; Kaźmierczak and Maszewski 1998). Biological and microchemical tests evidenced the acidic character of ACCF combined with its apparent ability to form cross-links with histones and DNA-histone complexes. Moreover, the fluorescence microscopy observations implied vesicular uptake and endocytosis-mediated distribution of rhodamine-isothiocyanate labeled ACCF-conjugates. All these data and the cell cycle-related effects evidenced previously in plants and human cells, including remarkable similarities to phenotypic alterations manifested in a.f. of *Chara*, stimulated our present investigations aimed at determining the metabolic changes of ACCF-treated root meristems cells of *M. noctiflorum.*

Materials and methods

Preparation of ACCF: Male sex organs containing a.f. at the final stages of cell proliferation (before their entrance into spermiogenesis) were selected and isolated from apical parts of *Chara tomentosa* L. f. *vulgaris* Mig. Preparation of antheridial extracts and purification of ACCF was performed according to methods described by Kaźmierczak and Maszewski (1998), with some minor modifications. In brief, a.f. obtained from maturing gametangia (3 g f.m.) were homogenized in the ice-cold mortar with 10 cm^3 of 50 mM Tris-HCl buffer (THB), pH 7.5 and centrifuged (4 000 *g*) at 4 °C for 20 min. To precipitate proteins, 4 vol. of acetone were added and, after chilling at -20 °C for 30 min, the probes were sedimented at 4 °C for 30 min, dried under vacuum and dissolved in the same medium. Following elution with THB on *Sephadex G-75,* the obtained samples indicating biological activity of ACCF were used for further experiments at the final concentration of about 20 μg cm^{-3} (determined by the method of Bradford 1976).

Plants: Seedlings of *Melandrium noctilorum* L. were grown on moist blotting paper at 20 - 22 °C in the dark. For all experiments intact plants with roots 1.5 - 2.0 cm in length were used for incubations. Since a number of preliminary examinations have shown that neither peptidic extracts made of vegetative parts nor extracts obtained from young male sex organs of *Chara* indicate biological activities of ACCF, the control incubations were made in THB, as it was the case in our previous studies (Maszewski 1995, Kaźmierczak and Maszewski 1997, 1998). Pretreatments in aerated solutions of ACCF and 5mM THB (control samples), before incubation in radioactive media or fixation adopted for Ag-NOR procedure, were performed throughout 24 h, at room temperature.

Autoradiography: Incubations in ^{3}H-uridine and ^{3}H-leucine (2.775 MBq cm^{-3} and 1.85 MBq cm^{-3}, respectively; *Amersham*, Buckinghamshire, England) were carried out for 60 min each and, after brief washing with running water, the plants were fixed in cold Carnoy's mixture for 1 h, washed thoroughly in ethanol and stained with Schiff's reagent according to standard methods (*e.g.* Maszewski and Kołodziejczyk 1991). Squash preparations were covered with *Ilford L4* nuclear emulsion, exposed for 14 (^{3}H-uridine) or 25 d (^{3}H-leucine), and the developed autoradiograms were mounted. In order to select G1, G2, and endopolyploid cells, nuclear DNA contents were evaluated by means of cytophotomery using the *Jenamed-2* microscope (*Carl Zeiss*, Jena, Germany) with the computer-aided *IMAL-512* system (*IMAL Co.*, Lódź, Poland) for image analysis. Absorbance in Feulgen-stained samples was measured at 565 nm.

Ag-NOR staining: The observations of nucleoli were carried out using a highly reproducible silver-staining technique for visualizing NORs, initially recommended by Goodpasture and Bloom (1975) and modified according to Howell and Black (1980). Seedlings were prefixed for 10 min in Sörensen's phosphate buffered (pH 7.2) 2 % glutaraldehyde, and then finally fixed in ethanol-acetic acid mixture (3:1, v/v) for 5 min at 4 °C. After fixation, the material was washed many times with ethanol, rehydrated, rinsed in distilled water, and placed into a solution containing one part of 2 % gelatine in 1 % formic acid and 2 parts of a 50 % $AgNO_3$ solution. Following Ag-staining at 60 °C for 7 min, plants were rinsed with many changes of distilled water and soaked for 10 min in a 5 % thiosulphate solution. After washing in water, apical root segments (1 mm long) were isolated, squeezed onto slides and frozen in a drop of 40 % acetic acid at -70 °C. Then, coverslips were removed, slides were dehydrated, dried and mounted. Nucleolar profile areas were measured using the computer-aided *IMAL-512* system.

Quantitative analyses for each experimental series were carried out by counting representative probes (≥ 50 cells per stage).

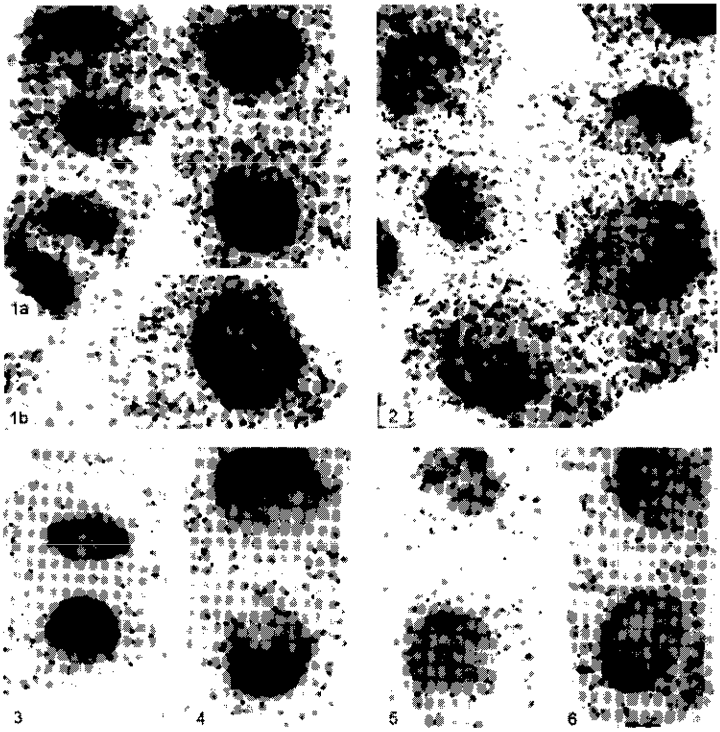
Results

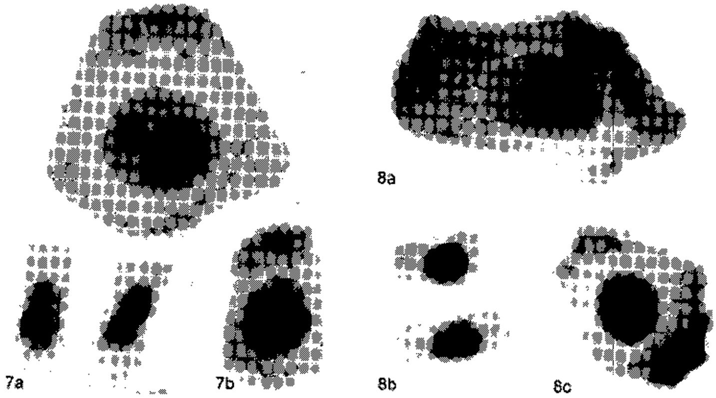
In order to avoid methodological problems connected with the natural heterogeneity encountered in root meristems of *M. noctiflorum*, autoradiographic analyses of ^{3}H-uridine (Figs. 1, 3, 4) and ^{3}H-leucine (Figs. 2, 5, 6) incorporation have been combined with Feulgen cytophotometry of nuclear DNA contents, allowing thus for selection of two distinct populations representing G1 and G2 phases of the cell cycle and endopolyploid cells having nuclear DNA content increased up to about 8C level. Application of radioactive markers during 1 h incubation resulted in a relatively uniform distribution of silver grains over the nuclear and cytoplasm areas, reflecting both net accumulation and translocations of molecules synthesized within particular compartments of the cell (Figs. 1 - 6).

Approximated selection of the G1, G2, and endopolyploid cells stained using the Ag-NOR method (Figs. 7 - 8) was made according to morphometric measurements of nuclear profiles.

Influence of ACCF on ^{3}H-uridine incorporation: Under the control conditions (Fig. 1), mean radioactivity calculated per nuclear unit surface area in the G2 phase cells is about 23 % greater than in the G1 phase cells (Fig. 9*a*). Since the actual nuclear profiles vary among the two analysed populations by a factor of 1.6, the estimated total transcriptional activity, expressed as the intensity of ^{3}H-uridine incorporation per nucleus, reveals a close correlation with the DNA C-values (Table 1). Concurrently, the G1-to-G2 phase transition brings about no significant change in the mean number of silver grains visualized above the unit surface area of the cytoplasm (Fig. 9*a*).

A 24 h preincubation in ACCF results in a marked decrease in the intensity of ^{3}H-uridine incorporation, evidenced both within the nucleus and the cytoplasm (Figs. 3 and 4). As compared with the control plants, the G1 and G2 phase cell nuclei in ACCF-treated root meristems of *M. noctiflorum* indicate about 90 % inhibition of transcription (Fig. 9*a*, Table 1). Concurrently, the average number of grains counted over the cytoplasm surface unit area decreases to 9.9 (G1) and 15.7 % (G2) of the control values. A similar extent of reduction in the intensity of ^{3}H-uridine





Figs. 1 - 6. Autoradiograms of cells from the apical root zone in *M. noctiflorum* seedlings following labeling with ^{3}H-uridine (1*a*,*b*, 3, 4) and ^{3}H-leucine (2, 5, 6) in the control conditions (1*a* - G1 and G2 cells, 1*b* - cell with 8C DNA content; 2 - cells in various phases of the cell cycle) and after preincubation with ACCF (3, 5 - G1 cells; 4, 6 - G2 cells). × 1300.

Figs. 7 - 8. Ag-NOR staining of cells from the apical root zone of *M. noctiflorum* in the control conditions (7*a*,*b*) and after preincubation with ACCF (8*a*-*c*). Endopolyploid (8C) cells are shown in the upper part of Fig. 7*a* and in Fig. 8*a*, G1-phase cells are presented in the lower part of Fig. 7*a* and in Fig. 8*b*, and G2-phase cells are shown in Fig. 7*b* and Fig. 8*c*. × 1200.

incorporation has been evaluated in cells with the nuclear DNA contents increased up to about 8C value (Table 1).

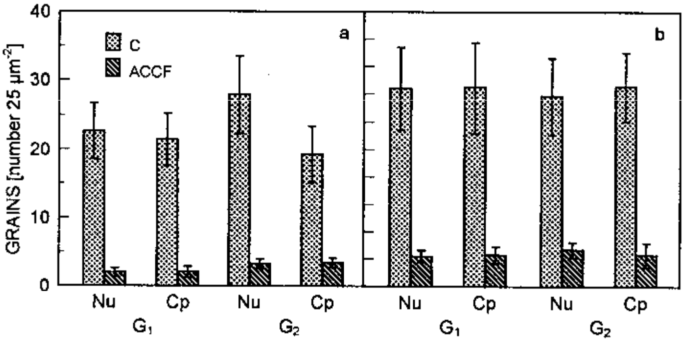


Fig. 9. Incorporation of ^{3}H-uridine (*a*) and ^{3}H-leucine (*b*) into nuclei (Nu) and into cytoplasm (Cp) of the G1- and G2-phase cells from the control (C) and ACCF-treated (ACCF) roots of *M. noctiflorum*.

Table 1. Transcriptional activity (^{3}H-uridine incorporation; number of silver grains) and the total nucleolar profile surface areas (nucleoli; μm^2; data compiled on the basis of result presented in Figs. 10 and 11) calculated per nucleus in the G1 (2C), G2 (4C) and endopolyploid (8C) cells in the control and ACCF-treated roots of *M. noctiflorum* (apical 1 mm-long segments).

DNA content		2C	4C	8C
^{3}H-uridine	control	57.2 ± 10.4	112.9 ± 39.5	180.7 ± 41.0
	ACCF	4.9 ± 1.5	12.9 ± 2.7	23.5 ± 5.9
Nucleoli	control	15.4	17.1	21.2
	ACCF	8.6	13.1	18.4

Influence of ACCF on ^{3}H-leucine incorporation: Although there is a clear general agreement between the kinetics of transcription (Figs. 1, 3, 4) and translation (Figs. 2, 5, 6), the relative differences between the intensities of ^{3}H-leucine incorporation into the control and ACCF-treated root cells appear slightly less pronounced. Mean levels of radioactivity, estimated autoradiographically in number of silver grains per unit surface area of cytoplasm, vary 6.3- and 7.1-fold (84 and 86 % inhibition) in the G1 and G2 cells, respectively (Fig. 9*b*). Concurrently, nearly proportional decreases in radioactivity have been found above the nuclear areas. ^{3}H-leucine incorporation into the nuclear and cytoplasmic areas of ACCF-treated cells with 8C DNA levels were found to be about 18 and 14 % of the control, respectively (Table 1).

Influence of ACCF on the nucleolar patterns: The maximum number of nucleoli in post-telophase cell nuclei in root meristems of *M. noctiflorum* was estimated to be 6, yet some extra Ag-stainable dot-like areas (reaching only a fraction of 1 μm in diameter) were frequently observed within the nucleus and the cytoplasm. The distribution patterns of interphase cells with different numbers of nucleoli vary between the control (Figs. 7*a, b*) and experimental series (Figs. 8*a-c*) according to the ranges presented in Fig. 10. Comparatively, in the ACCF-treated meristems an increased portion of cells assigned to the G1 phase (Fig. 10*a*) develops 1 or 2 nucleoli and a considerably lower fraction of cells is characterized by the presence of 3 - 6 nucleoli per nucleus, which decreases their mean number by about 20 %. A similar, yet considerably less pronounced tendency is evidenced in nuclei assigned to the G2 phase of the cell cycle (Fig. 10*b*) and those indicating DNA contents raised up to the 8C level (data not shown).

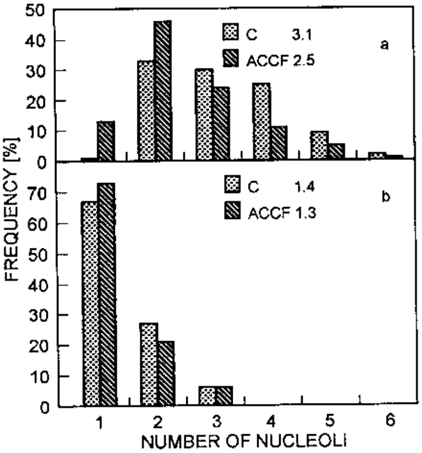


Fig. 10. Frequency distribution patterns of G1-phase (*a*) and G2-phase cells (*b*) with different numbers of nucleoli in the apical root zones of *M. noctiflorum* from the control (C) and ACCF-treated (ACCF) plants. Means are 3.1 and 2.5 for control and ACCF-treated plants in G1-phase and 1.4 and 1.3 for those in G2-phase, respectively.

The ACCF-treatment brings about a considerable reduction of nucleolar sizes in all categories of cells encountered within the 1-mm-long apical root segments of *M. noctiflorum* (Fig. 11*a*,*b*). Under the control conditions, the profile surface areas in about 50 % of nucleoli in the G1-phase nuclei range between 2.1 and 5.9 μm^2 (Fig. 11*a*). In ACCF-treated plants, an increased proportion of small-sized nucleoli (up to 1.9 μm^2) and the decreased fractions of larger nucleoli yield roughly 30 % reduction of the mean nucleolar profile area. A similar overall change is also evident in the G2 fraction of cells (Fig. 11*b*); in this case, however, reduction of the mean nucleolar surface area reaches merely some 20 %.

Considering a lowered number of Ag-stainable areas per cell nucleus in roots exposed to ACCF, the total nucleolar profile surface areas in G1 phase nuclei decrease to some 56 % of the control cells. An evident overall reduction of the total nucleolar areas is also found both in cell nuclei advanced in interphase (assigned to the G2 phase of the cell cycle) and in the endopolyploid cells. The mean total profile surface area estimated for nucleoli in the G2 phase nuclei was found 23.4 % lower in the ACCF-treated apical root segments than in the control plants (Table 1). A slightly smaller degree of reduction was evaluated for endopolyploid cells having their amounts of nuclear DNA increased up to about 8C value (data not shown).

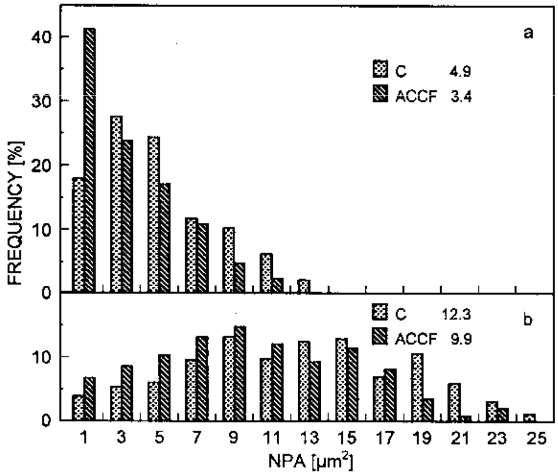


Fig. 11. Frequency distribution patterns of nucleoli indicating various profile surface areas (NPA; µm^2) in the G1-phase (*a*) and G2-phase nuclei (*b*) in the apical root zones of *M. noctiflorum* from the control (C) and ACCF-treated (ACCF) plants. Mean nuclear profile areas are 4.9 and 3.4 for control and ACCF-treated plants in G1-phase and 12.3 and 9.9 for those in G2-phase, respectively.

Discussion

Changes in chromatin structure exert a profound impact on many cellular processes, which become particularly evident in those kinds of events when extensive genetic rearrangement is needed to prescribe a new developmental program, or when complete cessation of nuclear activities is required to allow for a maximum efficiency in performing extremely specialized functions (Felsenfeld 1992, Grigoryev and Woodcock 1993). A vast amount of close correlations have been found previously between the decreases in sizes of spermatids, the durations of interphase periods and the many gradual metabolic changes that accompany progression of germ-line cells of *Chara* throughout the proliferative period of spermatogenesis. Repression of transcription during successive cell cycles (Kwiatkowska and Maszewski 1979) correlated with the increasing condensation of chromatin and progressive shortening of chromosomes, proceeds in proportion to the reduction of nuclear sizes (Olszewska and Godlewski 1972, Maszewski and Kołodziejczyk 1991), the decreased capacity to bind ^{3}H-actinomycin D (Olszewska 1974b) and the increase in size and number of chromocentres (Olszewska 1978).

Considering the numerous observations emerging from previous studies, it seems reasonable to assume that a sharp decrease of transcription evidenced in *M. noctiflorum* within all types of root cells exposed to peptidic factor derived from maturing antheridia of *Chara* results from its more or less direct influence on the physical structure of the nuclear genome. Any interaction affecting the intricate organization of chromatin or altering the packaging of DNA into nucleosomes can have large and versatile effects on gene activation. The mechanisms involved in such process may act *via* multiple factors, including histone-DNA positioning, boundaries defined by specific DNA conformation or protein binding (Workman and Buchman 1993, Lu *et al.* 1994). Whatever the actual mode of intermediary functioning at the molecular level, the influence of ACCF reveals no apparent specificity with respect to the stage of the cell cycle. However, the inhibitory efficiency of ACCF, pronounced slightly more at the G1- than in G2-phase or in cells with the DNA contents increased up to about 8C value, corresponds well with the preferential arrest of the cell cycle at the 1st Principal Control Point located at the G1/S boundary during interphase (Maszewski and Kaźmierczak 1995).

The impairment of transcription in root meristem cells of *M. noctiflorum* is closely correlated with a marked reduction of ^{3}H-leucine incorporation. The latter process is manifested by about 6-fold drop in number of silver grains evidenced over the nuclear and cytoplasm regions. Proportional decreases of ^{3}H-uridine and ^{3}H-leucine incorporation into both compartments allows to assume that in spite of an apparent overall deregulation of gene expression in ACCF-treated root cells, the mechanisms responsible for nucleo-cytoplasmic exchange remain undisturbed. A slight disproportion between the reduction of transcription (about 9-fold) and translation results, probably, from the occurrence of the long-living mRNA molecules continuously active within the cytoplasm.

A further obvious reflection of the modified quantitative physiology of ACCF-treated root cells, and one which correlates both with the decreased efficiency of transcription, translation, and with the innate processes evidenced in germ line cells of *Chara*, concerns the altered parameters of nucleolar organization. Reduction of total volumes of nucleoli, which occurs during every developmental stage of a.f., has been evidenced as a chain-like process supplied with multiple internal backup events typical for the degenerative self-regulating feedback system (Kwiatkowska and Maszewski 1985, Maszewski 1994). Assuming direct relationships between the amount of Ag-stainable proteins associated with nascent pre-rRNA molecules (such as nucleolin and C23; Herrera and Olson 1986, Roussel and Hernandez-Verdun 1994) and transcriptional activity of NORs (Moreno *et al.* 1989, 1990), morphometric analyses of nucleoli in root meristem cells of *M. noctiflorum* bring about further confirmation of the capability of ACCF to extensively rearrange the functional properties of the nuclear genome. Moreover, in agreement with the observations indicating close relationship between Ag-NOR areas and the dynamics of cell proliferation (Derenzini *et al.* 1994), our present results correlate well with the data showing the ability of ACCF to induce progressive inhibition of root elongation, reduction of mitotic indices and decreasing numbers of cells incorporating ^{3}H-thymidine (Maszewski and Kaźmierczak 1995).

A vast amount of information provides strong evidence that remodelling of nuclear structure throughout successive cell division cycles in a.f. of *Chara* represents an apparent morphological manifestation of the complex biochemical mechanisms raised to reset the ontogenetic clock by which germ line cells lose the competence to proliferate in association with induction of terminal transformation into spermatozoids (Maszewski 1994, 1995). The ability of antheridial peptidic extracts to exert remarkably similar effects in various unrelated cellular systems implicates that there may be a common causal principle underlying reorganization of chromatin and affecting its descendent activities. Certainly, there is a need to discriminate between the "programmed" nuclear inactivation in a.f. of *Chara* and the "incidental" rearrangements of chromatin induced by ACCF in heterogeneous systems of root meristem cells. Concurrently, however, several arguments encourage for considering metobolic changes in terms of such functional links (Kaźmierczak and Maszewski 1998). A highly negative charge, the presence of reactive sulphydryl groups associated with cysteine, and the ability to aggregate histones and DNA-histone complexes *in vitro* into condensed chromatin-like networks may implicate the capacity of ACCF to form cross-bridges that alter spatial design of nucleosomal DNA fibrils, and thus, impact on their competence to perform the ascribed roles efficiently. Such interpretation fits well previous data pointing to significant role of endogenous protein -SH groups in successive developmental stages of a.f. in *C. vulgaris* (Olszewska and Marciniak 1980).

The fundamental similarities between the "innate" processes within developing a.f. and the genetic repression induced by antheridial peptidic extracts in root cells of *M. noctiflorum* provide further positive evidence for the presence of a highly specific factor that contributes to nuclear re-patterning of differentiating germ line cells in male sex organs of *Chara*. Although such concept is based primarily on correlative data, further investigations may contribute to explain means by which the antheridial cells are driven towards the loss of their proliferative potential and finally, after having established haploid content of nuclear DNA, become permitted to traverse into spermiogenesis.

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