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The Plant Nucleolar Cycle Under Hypoxia

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Summary

Hypoxia disturbs the nucleolar cycle in *Allium cepa* L. meristems by diminishing the disorganizing stage and increasing the nucleologenesis time.

Though nucleolar remnants persist in the enlarged metaphase recorded under hypoxia, prenucleolar bodies appear at the same time than in control meristems if related to the timing of nucleolar envelope breakage. Such appearance is apparently independent of the chromosome condensation cycle, since it took place in anaphase and not in midtelophase as in controls.

Finally, the involvement of NORs in segregation is questioned since prenucleolar bodies are also segregated under hypoxia, both when integrated in the reforming nuclei or when dispersed in cytoplasm.

Keywords: Nucleolar cycle; *Allium cepa* L.; Hypoxia.

1. Introduction

Mitosis is a natural system for the splitting of the duplicated genome, which switches off most of the gene activity in a cell. Mitosis implies the later realignment of the genetic machinery when the sister cells are ready to initiate their new cycle. Nucleologenesis is a process which takes place in late telophase/early G_1 in *Allium cepa* L. root meristems (GIMÉNEZ-MARTÍN *et al.* 1974) with the involvement of the so-called prenucleolar bodies (MCCLINTOCK 1934, GUTTES *et al.* 1968, STOCKERT *et al.* 1970 a, LAFONTAINE and LORD 1973) whose appearance precedes both any detectable nucleolar transcription (MORCILLO *et al.* 1976) and the appearance of a true mature organized nucleolus (GIMÉNEZ-MARTÍN *et al.* 1977).

The coalescence of some of the prenucleolar bodies on the nucleolar organizer region (NOR) seems to be an early step in nucleolar formation and perhaps in the re-start of the rDNA transcription, re-start which is a prerequisite for completion of nucleologenesis to occur (MORCILLO and DE LA TORRE 1979).

Prenucleolar bodies may be formed by the basic components of the nucleolar substructure. The incorporation of RNA molecules which have been synthesized during G_2 of the previous cell cycle in the new nucleolus has been demonstrated (LEPOINT and GOESSENS 1978). The study of such prenucleolar bodies under electronmicroscopy has shown them to be fibrillar (LAFONTAINE and CHOUINARD 1963, STEVENS 1965, MORENO DÍAZ DE LA ESPINA *et al.* 1976, PLOTON and GONTCHAROFF 1979) or fibrillo-granular in composition (STOCKERT *et al.* 1970 a, SACRISTÁN-GÁRATE and STOCKERT 1974).

The present work tries to relate nucleoli and prenucleolar bodies in meristems under hypoxia, when RNA synthesis and processing are nearly halted secondarily to a shift in the energy charge (ASPART *et al.* 1983).

2. Material and Methods

Onion bulbs of *Allium cepa* L. were kept with their bases submerged in water at 25 ± 0.5 °C in cylindrical glass receptacles. Control conditions were obtained by bubbling air at a rate of 10–20 ml/minute.

2.1. Hypoxia Treatment

When the sprouted roots were about 3 mm long they were placed in water previously boiled for 30 minutes in which 1 cm thick layer of liquid paraffin was made to float.

They were kept in such conditions for 24 hours. Recovery was

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obtained by placing for 6 hours the bulbs with their attached roots in receptacles where air bubbling (10–20 ml/minute) was going on. Control roots were obtained before treatment in the same roots. Treated roots were studied after 6, 12, 18, and 24 hours hypoxia. Recovery was obtained 6 hours after returning the roots at the air bubbling conditions.

2.2. Staining

Roots were fixed in a 10% formalin—1% hydroquinone mixture for one night at 4 °C. Then they were washed in distilled water three times in half and hour. They were stained with 2% $AgNO_3$ at 70 °C overnight, followed by a new washing in distilled water and 1–2 hours in the formalinhydroquinone mixture again. After washing the 2nd terminal mm segments of root apices were squashed and observed under Zeiss photomicroscope.

2.3. Electronmicroscopy Technique

Roots were fixed in 3% glutaraldehyde in a 0.05 M cacodylate buffer at pH 7.0 for 2 hours at room temperature, and postfixed in 1% osmic acid for 1 hour in the same buffer. Dehydration was carried out with an ethanol series, followed by embedding in Epon 812. Ultrathin sections obtained with an LKB ultratome were stained with uranyl acetate and lead citrate. A Philips 300 electron microscope at 60 kV was used for getting ultramicrographs.

3. Results

The kinetic analysis of the nucleolar cycle in control and hypoxic conditions have been studied in meristems growing under steady state conditions. In control meristems, the disorganization of nucleolus starts earlier than metaphase (Fig. 1) and ends with the triggering of such mitotic phase. Reappearance of prenucleolar bodies, which marks the initiation of nucleologenesis, occurs late in the chromosome cycle (*i.e.*, in telophase) and the nucleologenesis is over only during G_1 of next cycle. The end of nucleologenesis is considered to occur when no prenucleolar bodies are farther seen.

Under hypoxia, the relative timing of metaphase in the cycle enlarges. Disorganization of nucleoli occurs in a shorter time than in control conditions but with a displacement of its initiation time which occurs closer to metaphase start. Moreover, the breaking up of the nuclear envelope does not involve the fast disappearance of nucleolar remnants. Instead, nucleolar remnants are detected on some metaphase plates.

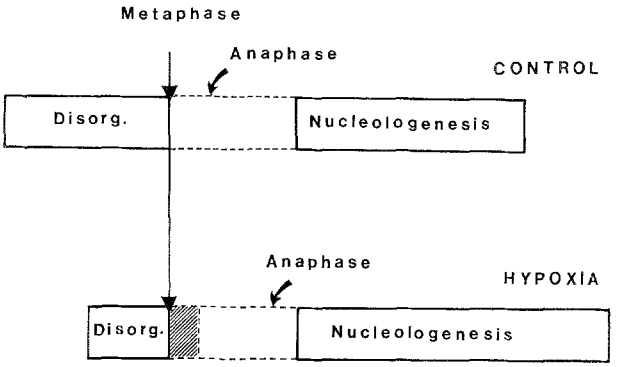

Fig. 1. Diagram showing the relative duration of the phases of the nucleolar cycle in both control and hypoxic conditions. Such relative durations are estimated from the recorded frequencies in both sorts of meristems. Fitting of both cycles has been accomplished in relation to the metaphase start (taken as the time when nuclear envelope breaks down). The relative duration of metaphase (*M*) in both situations has also been drawn. It is 2.5 times larger under hypoxia. Observe that the initiation of nucleologenesis occurs in nearly identical time in relation to metaphase start. Since metaphase is enlarged under hypoxia, prenucleolar bodies (PBs) should appear earlier in the post metaphase stages. This is confirmed by visual observation. The appearance of PBs is hardly modified by the persistence of nucleolar remnants in metaphase

Curiously enough, the reappearance of prenucleolar bodies occur in rather similar time if related to the metaphase start, as if it were linked to such process but not to anaphase triggering (end of metaphase). Such advancement would imply the appearance of prenucleolar bodies on anaphase chromosomes.

Nucleologenesis itself occupies a larger fraction of cycle, suggesting some energy-dependence of this process.

Fig. 2. *a* Light microscopy of anaphase of a control meristem. No silver stained material is seen on these chromosomes. *b* Control telophase. Arrows point out some silver stained dots on chromosome arms. This stage represents the start of nucleologenesis. *c* Control telophase. Small arrows point out silver positive prenucleolar bodies. Thick arrows point out the nucleolar organizer regions where new nucleolus is being assembled. Nucleologenesis is going on. *d* and *e* Two focal planes of a single cell in late telophase. Prenucleolar bodies (arrows) are dispersed in the sister nuclei. New forming nucleoli are clearly distinguished. Nucleologenesis still is going on, since there are still PBs. *f* and *g* Two nuclei with a pair of mature nucleoli in each nucleus. Nucleologenesis has finished since no PBs are observed. *h* Electronmicrography of the nucleolus of a control cell. Fibrillar (*F*) and granular (*G*) components are evident in such a nucleolus. The arrow points out a vacuole with a spot of dense material, probably dense chromatin. *i* Sister nuclei in early interphase in meristems under hypoxia. Nucleolar segregation is clearly seen, characterized by the appearance of a highly positive dense core surrounded by a light silver stained rim. *j*. Detail of a nucleolus segregated under hypoxic conditions. Dense and light areas will be seen to correspond to the fibrilllar (*F*) and granular (*G*) regions of nucleolus, defined under electron microscopy. The dense core often reaches the nucleolar surface in some places. Bar represents 10 μm. *k* Detail of a nucleolus segregated by hypoxia. The fibrillar component (*F*) stays in a central position, sometimes with vacuoles which do not contain any dense material this time. The granular component forms a peripheral layer on segregated nucleoli

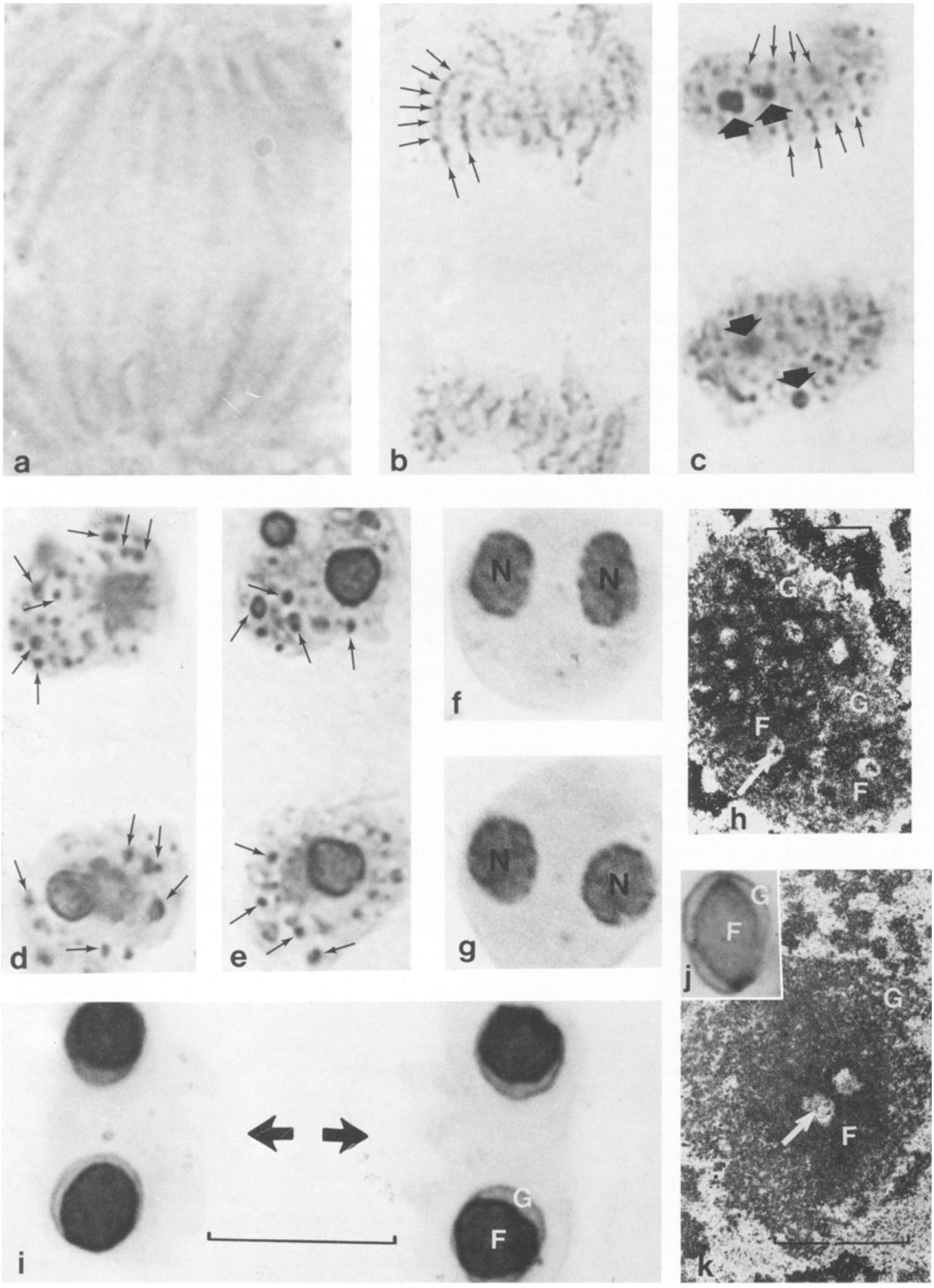

Fig. 2

When looking at silver stained preparations of control meristems we can see how anaphase chromosomes hardly contain any particulate stained material (Fig. 2 *a*) while argyrophilic bodies are seen in telophase along most if not all chromosomes arms (Fig. 2 *b*). More advanced telophases allow to see prenucleolar bodies plus the incipient new nucleoli which appear as larger prenucleolar bodies on NORs (arrows in Fig. 2 *c*). Prenucleolar bodies of different but similar sizes are observed in late telophase/early interphase stage. Two focal planes of the same cell are shown in Figs. 2 *d* and *e*.

Newly formed nucleoli in interphase nuclei whose nucleologenesis is over are observed in nuclei which do not contain prenucleolar bodies at all (Figs. 1 *f* and *g*). Electron microscopy of such mature nucleoli shows their compound nature with both fibrillar and granular components (*F* and *G* in Fig. 2 *h*). The arrow in this Fig. 2 *h* points out a lacunar space with dense content.

When meristems are grown under hypoxia, the phenomenon of nucleolar segregation takes place, *i.e.*, nucleoli appear as formed by a lighter halo surrounding a highly argyrophilic core (Figs. 2 *i* and *j*).

Under the electron microscope, such segregated nucleoli show how the more argyrophilic zone corresponds to the fibrillar component (*F*) while the halo corresponds to the granular one (*G*) (see Fig. 2 *k*). Vacuoles without dense inner content (arrow in Fig. 2 *k*) are often seen.

Under hypoxic conditions, prenucleolar bodies appear in chromosomes in anaphase or early telophase (Figs. 3 *a–c*). Two focal planes of the same cell are those of Figs. 3 *b* and *c*. Forming nucleoli are seen on the NORs of the pair of NOR-bearing chromosomes in symmetrical places of both sister chromosomal groups. Hypoxia also produces the appearance of prenucleolar bodies on cytoplasm of anaphase cells, distant apart from chromosomes (arrows in Fig. 3 *d*).

When looking at prenucleolar bodies in telophase of cells growing under hypoxia conditions, one striking feature of such prenucleolar bodies is evident: they are compound in nature as nucleolus itself and they show segregation of both components (Figs. 3 *e, f,* and *i*). The darker zone of such prenucleolar bodies, whose density seems to correspond to the inner core of segregated nucleoli, is often located in a peripheral position, either as a single mass or as two or more particulate regions (see arrows in Figs. 3 *e* and *f* which represent two focal planes of the same cells and insert in Fig. 3 *f*). In contrast, darker regions in segregated nucleoli are rather central, though they can reach the periphery in what seems to be like crater mouths (Fig. 3 *h*). Very often, when the pair of nucleoli which characterizes the diploid nuclei in onion are close, the granular portion (less stained region) of such nucleoli can become fused (arrow in Fig. 3 *g*).

Finally, prenucleolar bodies which are not included in the newly formed telophase nuclei are free in the cytoplasm with clear segregation of their two components (empty arrows in Fig. 3 *i*).

4. Discussion

Hypoxia produces a drop in the energy charge of the cell and in its general metabolic activity (ASPART *et al.* 1983) apart from a shift in the pattern of proteins synthesized (SACHS *et al.* 1980).

Hypoxic conditions in onion meristems allowed us to see that metaphase (with the assembly of microtubules and microtubules kinetochores), but not any of the other mitotic phases, is a process highly dependent on the cell's continuous supply of energy.

In relation to nucleolus, hypoxia produces its segregation in interphase and the lengthening of nucleologenesis, as it could be expected from a depressed rate of

Fig. 3. *a* Anaphase chromosomes in meristems under hypoxia. The silver stained dots which correspond to the prenucleolar bodies are already evident on still enlarged chromosomal arms (arrows). Nucleologenesis has already started. Bar represents 10 μm. *b* and *c* Two anaphases in hypoxic conditions. Not only prenucleolar bodies but nucleolus itself is being assembled on NORs (thick arrows). *d* Cell in anaphase under hypoxia. Apart from prenucleolar bodies and incipient nucleolus, silver positive material appear on cytoplasm (arrows), far away from chromosomal arms. *e* and *f* Two focal planes of a telophase cell under hypoxia. Nucleologenesis is still going on. Prenucleolar bodies (thin arrows) and growing nucleoli (thick arrows) are seen. Observe that segregation of dark and light stained material is seen both in nucleoli and in prenucleolar bodies. Such prenucleolar bodies appear to have a larger amount of granular (light stained area) than fibrillar component. The fibrillar component (dark stain) is often peripheral and multiple in the prenucleolar bodies, at variance with the situation observed in the nucleolus. *g* Detail of a segregated nucleolus in a nucleus where the pair of nucleoli has spontaneously fused. The fusion of the granular but not of the fibrillar component is evident (arrow). *h* Detail of a segregated nucleolus (*N*) under hypoxia. Arrows point out a large prenucleolar body with three dots which are highly contrasted by silver. Observe that they remain as discrete spots in the periphery of the less stained mass. *i* Hypoxia produces the dispersion of prenucleolar bodies in cytoplasm. Such prenucleolar bodies are not integrated in the reforming nuclei. Empty arrows point out three of such prenucleolar bodies. Dark and light stained regions keep a relationship which differs from that seen in segregated nucleoli. Here the highly stain material occupies a peripheral location and may be separated in more than a single mass

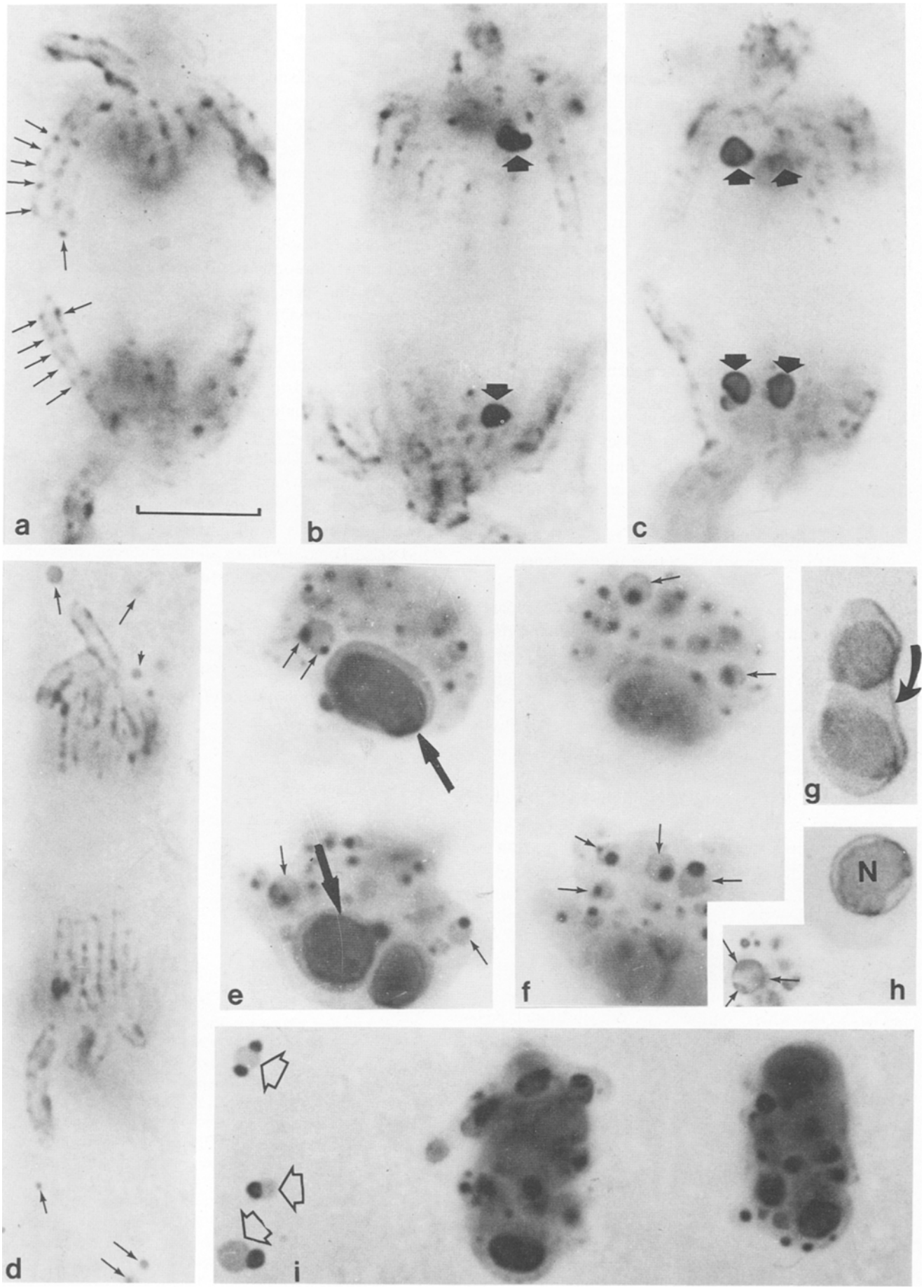

Fig. 3

RNA synthesis (STOCKERT *et al.* 1970 b) secondary to the lowering in energy charge.

Nucleologenesis is a complex assembly process, with an initial step characterized by the appearance of prenucleolar bodies (PBs) on telophase chromosomes. Hypoxia makes evident that such appearance is neither related to a particular chromosomal condensation or to energy supply. The appearance of PBs appears to be triggered earlier in the cycle and probably in parallel with metaphase, since both processes are in a sequence, at a fixed temporal distance which is independent of the actual kinetics of the chromosomal cycle, or of the persistence of nucleolar remnants in metaphase (Fig. 1). The completion of nucleologenesis was seen to occur under hypoxia, suggesting that postmitotic cells are able to reinitiate transcription under low O_2 tension, since the assembly process is not finished without such reinitiation (GIMÉNEZ-MARTÍN *et al.* 1974, MORCILLO and DE LA TORRE 1979).

Segregation of both prenucleolar bodies and nucleoli confirm their compound nature in meristems under hypoxia. However such segregation was never evident in PBs under inhibition of transcription. This apparent contradiction may be explained if only PBs produced under hypoxia have an important amount of granular components which will make segregation detectable.

This hypothesis is sustained by two observations: from the two argyrophilic proteins C 23 and B 23 only the first (C 23) was immunologically detected both in nucleoli and PBs (OCHS *et al.* 1983) and PBs under an inhibitor of RNA synthesis were indeed shown to be composed of both granular and fibrillar part at the electron microscope level, but the granular portion was in minority (SACRISTÁN-GÁRATE and STOCKERT 1974). The presence of such an important amount of granular components in PBs under low O_2 tension is consistent with the reported halt in maturation steps of pre-ribosomal RNA brought about by anoxia (ASPART *et al.* 1983).

The NOR, where synthesis of pre-ribosomal RNA takes place, always is surrounded by fibrillar portion of nucleoli where pre-rRNA accumulates, while granular portion which surrounds the fibrillar one contains maturation derivatives of such pre-rRNA (GRANBOULAN and GRANBOULAN 1965, LA COUR and CRAWLEY 1965). Hence, the disposition of NOR implies that of other nucleolar components.

Natural and induced segregation always involves a specific disposition of NOR, which secondarily induces that of apposed fibrillar and granular components (GÍMENEZ-MARTÍN *et al.* 1977). Both components appears topographically separated, the granular one in a peripheral position as a rather eccentric crown.

Thinking of NOR as a "puff" structure (VINCENT 1955, LA COUR and WELLS 1966), induced segregation was postulated to occur through retraction (collapse) of NOR looped structure towards the chromosome axis, with the result of bringing the other nucleolar components—fibrillar and granular—to a new positional redistribution (FERNÁNDEZ-GÓMEZ *et al.* 1972). However, the segregation of PBs occurs without any involvement of NOR. Hence, it can be postulated that PBs themselves carry out structural information which leads to a similar pattern of segregation. Here granular components tend to fuse in a central position while fibrillar ones remain as particulate components in the periphery of PBs.

Moreover, prenucleolar bodies do indeed seem to carry out information which allows both their auto-assembly as well as some limited assembly with NOR when nucleologenesis is artificially lengthened (MORCILLO and DE LA TORRE 1980). In all the observed cases when two or more PBs fuse, such fusion occurs through their granular components.

Finally, the appearance of prenucleolar bodies in cytoplasm resembles the observation carried out after a sublethal thermal shock (DÍEZ *et al.* 1971). In both situations an impairment of the respiration function may be implicated.

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