NUCLEAR BODIES IN THE MATURING EGG CELL OF A FERN, PTERIDIUM AQUILINUM

P. R. BELL
Department of Botany and Microbiology, University College London, Darwin Building, Gower Street, London WC1E 6BT, U.K.

SUMMARY

Nuclear bodies about 250 nm in diameter, and with a strong affinity for uranium and acriflavine, appear in the nuclei of maturing egg cells of Pteridium. Many enter well-defined evaginations of the nucleus. The nuclear bodies are almost wholly digested by Pronase, but are resistant to ribonuclease and deoxyribonuclease. Radioactive labelling gives no evidence of the presence of nucleic acids, but X-ray microprobe analysis indicates phosphorus. It is concluded that the bodies consist entirely of acidic protein, possibly phosphorylated. This protein may be a structural component of the nucleus, temporarily displaced and aggregated as a consequence of the fine dispersal of the chromatin.

INTRODUCTION

During maturation of the egg cells in all leptosporangiate ferns so far examined there is extensive interaction of nucleus and cytoplasm (Bell, 1975). Evaginations issue from the main body of the nucleus, but remain connected by channels rarely exceeding 50 nm in internal width. Pteridium aquilinum, in which the evaginations are relatively simple in form, has been studied in greatest detail. In this species the evaginations regularly contain an aggregate of dense material, which appears strongly electron-opaque after conventional staining with heavy metals (Bell, 1970). These aggregates arise close to the nucleolus and have been called micronucleoli (Tourte, 1968). Evidence of their nucleolar nature is, however, wanting and they are better referred to as nuclear bodies (Bell, 1975). The establishment of the chemical nature of the material of the nuclear bodies is essential for understanding the significance of the nuclear behaviour, particularly since this may coincide with the entry of messengers into the cytoplasm in preparation for subsequent sporophytic growth (Bell & Duckett, 1976).

There is a striking resemblance between the staining of the nuclear bodies and that of the nucleolus, ribosomes and chromatin. The larger nuclear bodies, for example, are visible in the light microscope, and they can be seen to have a strong affinity for the basic stain Azure B. This staining behaviour made it seem plausible that a nucleic acid was present. Nevertheless, extensive and critical labelling experiments with tritiated thymidine failed to substantiate an earlier view that the bodies contained DNA (Bell, 1979).

The following investigation was undertaken to investigate the chemical nature of the nuclear bodies in greater depth.
MATERIALS AND METHODS

Dehydration without using organic solvents

Thin slices (about 0.5 mm in width) were cut from the archegoniate region of gametophytes of *P. aquilinum* and fixed in 4.5 % glutaraldehyde (TAAB, U.K.) in 0.05 M-phosphate buffer (pH 6.9) for 5 h at room temperature. After several changes of ice-cold buffer overnight, the slices were transferred to a 1:3 mixture of Durcupan A (Fluka AG, Buchs, Switzerland) and distilled water, and left for 24 h. Subsequent transfers were to mixtures of the same compound in the proportions 1:1 and 3:1, and ultimately to pure Durcupan A. Infiltration with pure Durcupan A was continued in a desiccator with two changes for 3 days. The material was next transferred to 3:1, 1:1 and 1:3 mixtures of Durcupan A and Epon (Shell, U.K.), and final embedding was in pure Epon. Egg cells at suitable stages of development were found by 4 µm sectioning and remounted for fine sectioning by the technique of Woodcock & Bell (1967). Silver sections were stained with saturated aqueous uranyl acetate.

Ribonuclease extraction followed by acriflavine and phosphotungstic acid staining

After fixation in glutaraldehyde and washing, one half of the slices of gametophyte were transferred to a solution of ribonuclease (1 mg/ml) in 0.05 M-phosphate (pH 7.0) buffer and incubated for 2 h at 35 °C. The remaining slices were incubated in buffer alone. After rinsing in buffer the slices were transferred to a solution (0.5 X 10^-3 M) of acriflavine (purified according to the method of Albert, 1966), also in phosphate buffer. The material was left overnight at 4 °C and subsequently washed for 48 h with several changes of ice-cold buffer. The slices were then dehydrated in acetone and embedded in Durcupan ACM (Fluka AG, Buchs, Switzerland). Egg cells at the appropriate stages of development were located and remounted for thin sectioning, as described above. Silver sections were stained with 1 % aqueous phosphotungstic acid for 15 min.

Enzyme extraction and frozen sections

Frozen sections were prepared and treated with enzymes according to the method of Sheffield, Laird & Bell (1983). Ribonuclease was used at a concentration of 1 mg/ml in 0.05 M-phosphate buffer (pH 7.0), deoxyribonuclease (Worthington, U.S.A.) at 1 mg/ml in 0.005 M-MgSO₄ brought to pH 6.8 with 0.04 M-Na₂HPO₄, Pronase (BDH, Poole, U.K.) at 1 mg/ml in 0.1 M-Tris (pH 7.2). All digestions were carried out for 1 h at 35 °C. Each control was incubated in the respective enzyme medium alone. A few sections were incubated in Pronase and ribonuclease sequentially, accompanied by the appropriate double control. Silver sections were stained with uranyl acetate alone, except after Pronase extraction when uranyl acetate was followed by lead citrate.

Radioactive labelling and autoradiography

Actively growing gametophytes of *P. aquilinum* in pure culture were transferred for 8 h to normal medium (Moore, 1903) supplemented with 40 µCi/ml tritiated uridine (New England nuclear, sp. act. 4.13 Ci/mmol). For arginine labelling the gametophytes were transferred to normal medium supplemented with 10 µCi/ml L-[5(n)-3H]arginine monohydrochloride (Amersham, sp. act. 22 Ci/mmol) for 2 days to saturate the amino acid pool, and subsequently grown for 4 days on normal medium. In both instances fixation was as for normal electron microscopy, followed by dehydration in acetone and embedding in Durcupan ACM. Electron microscopic autoradiographs were prepared as described by Schedlbauer, Cave & Bell (1973).

Radioactive labelling with tritiated actinomycin D (Amersham, sp. act. 9.1 Ci/mmol) was carried out on material fixed normally. After washing, the material was then returned to buffer and washed for 24 h, osmicated (1 % aqueous for 2 h) and embedded. Electron microscopic autoradiographs were then prepared as above.

X-ray microprobe analysis

Analyses were carried out on material fixed in the normal manner, but with the substitution of 0.1 M-sodium cacodylate buffer (pH 7.2) for phosphate. Sections were cut at 200 nm and...
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examined in an AEI-CORA analytical microscope in conditions similar to those used by Sigee & Kearns (1981).

RESULTS

Removal of water by progressive replacement with water-soluble resin resulted in electron micrographs with less contrast, but the nuclear bodies were particularly conspicuous (Fig. 1). Within the evaginations, their diameters were of the order of 250 nm compared with, at the most, 200 nm after normal dehydration. Although the nuclear bodies, like the nucleolus, were only just detectable in the unstained sections, uranyl staining resulted in dense blackening. The electron opacity of the nuclear bodies after uranyl staining was often greater than that of the nucleolus (Fig. 1).

The nuclear bodies also had strong affinity for acriflavine and they acquired sufficient electron opacity to be seen without further treatment. Following phosphotungstic acid staining, however, the contrast was considerably enhanced (Fig. 2); the nuclear bodies again appearing blacker than the nucleolus. Following ribonuclease treatment the affinity of the nucleolus for acriflavine was strikingly diminished. That of the nuclear bodies was not notably affected (Fig. 3), although the material possibly became less coherent.

Results of the enzyme treatment of frozen sections were quite consistent. Twelve egg cells were examined in the definitive experiments after the technique had been developed. Ribonuclease and deoxyribonuclease produced no greater effect upon the material in the evaginations than extraction with medium alone (Figs 4, 5, 6). The

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Fig. 1. Portion of the nucleus and adjoining cytoplasm of a maturing egg dehydrated by progressive replacement of the water with water-soluble resin before final embedding in Epon. No osmication; section stained with uranyl acetate. nu, nucleolus. Arrows point to nuclear bodies that have entered evaginations. X40 000.

Fig. 2. Portion of the nucleus of a maturing egg cell in a thin slice of gametophyte extracted with ribonuclease medium (control); subsequently treated with acriflavine, embedded and sectioned. Section stained with phosphotungstic acid. nu, nucleolus. The arrow indicates a nuclear body. X75 000.

Fig. 3. As Fig. 2, but the egg cell was extracted with ribonuclease before acriflavine treatment. X75 000.

Fig. 4. Frozen section extracted with Pronase and ribonuclease media before inclusion in resin (double control). Thin section stained with uranyl acetate. The material in the nuclear evagination (arrow) is not detectably affected by the double extraction, but the distinctness of the ribosomes is diminished (cf. Fig. 5). X80 000.

Fig. 5. Frozen section extracted with deoxyribonuclease. Subsequent treatment as in Fig. 4. X80 000.

Fig. 6. Frozen section extracted with ribonuclease. Subsequent treatment as in Fig. 4. X100 000.

Fig. 7. Frozen section extracted with Pronase. Subsequent treatment as in Fig. 4, with the addition of lead staining to make membranes visible. n, main body of nucleus. X100 000.

Fig. 8. Autoradiograph of a portion of the nucleus of a maturing egg fixed after feeding with tritiated uridine. nu, nucleolus. The arrows indicate nuclear bodies. X25 000. Inset: the egg cell of which the autoradiograph was taken. The nucleolus appearing in the autoradiograph is the one to the right of the photomicrograph. X630.
Figs 1–3. For legend see p. 111.
Figs 4–8. For legend see p. 111.
effect of Pronase was in every instance decisive. Most of the contents of the egg cell were removed and the profiles of organelles were often distorted. No nuclear bodies were recognizable either in the main body of the nucleus or in evaginations (Fig. 7).

Following treatment with tritiated actinomycin D, autoradiographs showed that silver grains were frequently present above somatic nuclei. In the egg cell grains were scattered over the profile of the nucleus, and were occasionally present above mitochondria and plastids. In 30 autoradiographs obtained from five egg cells, all showing active formation of nuclear evaginations, no instance was found of silver grains associated with nuclear bodies either within the nucleus or in the evaginations. Autoradiographs taken after feeding with tritiated uridine showed extensive labelling of nucleolar material (Fig. 8), and scattered labelling of chromatin. The nuclear bodies in all preparations were unlabelled (Fig. 8, arrows). Very similar results were obtained after feeding with tritiated arginine.

X-ray microprobe analyses of the nuclear bodies in evaginations gave firm evidence of the presence of phosphorus, and less-convincing evidence of calcium. The emission spectra were otherwise insufficiently distinct from background to warrant drawing conclusions.

**DISCUSSION**

The present investigation makes clear that the principal, and possibly only, component of the nuclear bodies in the mature egg of *Pteridium* is highly acidic protein. The detection of phosphorus within the bodies with the X-ray microprobe suggests that the protein is phosphorylated, and this may account for the acidity. Free carboxyl groups may also be present. Although there are clear indications that the nuclear bodies arise adjacent to the nucleolus, the failure of the nuclear bodies, in contrast to the nucleolus, to show labelling when fed with tritiated arginine speaks against close similarity in their proteins. The nuclear bodies thus have no resemblance to the micronucleoli that occur in amphibian oocytes, since these contain both DNA and RNA (Miller, 1966). Nor are they identical to the nuclear bodies described in oocytes of the echinuroid worm *Urechis* (Das & Alfert, 1973). Although nucleic acids were absent from these bodies, the proteins, on the basis of their staining behaviour, were regarded as being similar to those of the nucleolus. It was proposed that in *Urechis* the formation of nuclear bodies, as opposed to micronucleoli, was a consequence of the failure of amplified ribosomal DNA to leave the principal nucleolus. Nuclear bodies also occur in the meristematic cells of plants, but these appear to resemble the nucleolus in composition (Luck & Lafontaine, 1982).

The chemical nature of the nuclear bodies in the egg cell of *Pteridium* makes it highly unlikely that they represent a stage in the transmission of transcribed information from nucleus to cytoplasm (Tourte, 1975). Since the production of nuclear bodies and evaginations continues with no evident loss of intensity in the presence of thiouracil (Bell, 1972), which is known to damage the capacity of the egg for subsequent sporophytic growth (Jayasekera & Bell, 1972), the physical aspects of the interaction seem independent of ribonucleic acid metabolism and the nature of the
Cytoplasm generated in the mature egg. The material in the evaginations may be structural proteins of ribosomes, some of which are acidic, but the striking increase in ribosome number has already occurred before the production of nuclear bodies begins (Cave & Bell, 1974). The significance of the evaginations may therefore lie elsewhere. The chromatin in the egg nuclei is more finely dispersed than at any other stage in the life-cycle. Phosphorylated non-histone proteins are known to be associated with chromatin in somatic nuclei (Cameron & Jeter, 1974; Kleinsmith, 1978). Some of these are concerned with chromatin structure and the assembly of nucleosomes (Laskey, Honda, Mills & Finch, 1974), and others with the control of transcription. It is possible that in the very peculiar nucleus of the fern egg these proteins either accumulate in excess, or are displaced from the chromatin, and aggregate to form nuclear bodies. These may then be eliminated from the nucleus, or temporarily sequestered within the nuclear evaginations. Alternatively, the dispersal of the chromatin may be accompanied by the breakdown of the nuclear matrix, also acidic in nature (Wunderlich & Herlan, 1977).

The fate of the nuclear bodies of *Pteridium* after fertilization has not been resolved. They have disappeared by the time the zygotic nucleus enters prophase (about 48 h after fertilization). Unfortunately, many more eggs are fertilized than develop (Bell, 1979). Although some of these eggs are clearly necrotic, others appear healthy. No features have yet been detected in young zygotes that separate the viable from the non-viable. Consequently, although profiles have been seen that could be interpreted as autolytic degradation of the evaginations and their contents, it is not certain that these are representative of developmental, as opposed to degenerative, changes. If the nuclear bodies are eventually released into the cytoplasm, and their functional significance has been correctly identified, it is conceivable that the constituent protein re-enters the nucleus as the chromatin condenses in readiness for prophase.

Temporary or permanent displacement of nuclear proteins may be a feature of other reproductive cells in land plants. During megasporogenesis in *Capella*, for example, active nuclear blebbing occurs in meiotic prophase. Both membranes of the envelope are involved, and the blebs contain electron-opaque material (Schulz & Jensen, 1981). The switches in gene activation leading to the phase changes in the life-cycle may be accompanied by extensive re-organization of the non-histone proteins, some of which aggregate to form the nuclear bodies.

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REFERENCES


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