A CYTOCHEMICAL AND RADIOAUTOGRAPHIC STUDY OF THE ULTRASTRUCTURAL ORGANIZATION OF PUFF-LIKE FIBRILLAR STRUCTURES IN PLANT INTERPHASE NUCLEI (ALLIUM PORRUM)

J. G. LAFONTAINE,* B. T. LUCK AND D. DONTIGNY
Department de biologie, Faculté des Sciences et de Génie, Université Laval, Québec, P.Q., G1K 7P4 Canada

SUMMARY

Loose, fibrillar, spherical structures have been observed during recent years in interphase nuclei of both animal and plant cells. These nuclear formations have been referred to as karyosomes, fibrillar bodies, micropuffs and centromeres. In order to gain further information on the nature of these structures, a cytochemical and radioautographic investigation was undertaken using plant meristematic cells (Allium porrum). For that purpose roots were fixed with either formaldehyde or glutaraldehyde in order to carry out cytochemical tests for DNA, RNA and proteins. Certain of the preparations were also first digested with DNase, RNase or proteinase K and then stained according to different procedures. Other specimens were labelled with thymidine for high-resolution radioautographic observations. Staining with diamino-benzidine (DAB) revealed that these nuclear puff-like formations consisted partly of a loose fibrillar meshwork containing nucleic acids. Part of this fine fibrillar reticulum persisted whether the preparations were digested with DNase or RNase before staining with DAB, thus indicating that these nuclear structures contained both DNA and RNA. The fact that these formations incorporate thymidine furnished additional support for the view that they correspond to specific chromosome segments. Staining with ethanolic phosphotungstic acid or digestion of specimens with proteinase K showed that these loose fibrillar structures also consisted of proteins.

Judging from their ultrastructure, their association with the chromatin reticulum as well as from their cytochemical characteristics, these nuclear formations most likely correspond to centromeres.

In view of the presence of DNA within these structures, it is possible to distinguish them from other equally spherical nuclear formations, observed in certain plant species, that have generally been referred to as karyosomes or micronucleoli and that appear to consist of ribonucleoproteins.

INTRODUCTION

During recent years, a number of different nuclear formations, usually spherical in shape and exhibiting a fibrillar ultrastructure have been reported in both animal and plant cells (refer to Risueño, Moreno Díaz de la Espina, Fernández-Gómez & Giménez Martín, 1978).

Although the fibrillar structures observed in plant cells vary in size to a significant extent, their organization is remarkably similar in the few species so far studied.

* To whom correspondence should be sent.
J. G. Lafontaine, B. T. Luck and D. Dontigny

Nonetheless, a variety of hypotheses have been put forward to account for their nature and their role within the nucleus.

The present study was undertaken, using various cytochemical techniques, with the purpose of characterizing further these nuclear structures and thus gaining additional data in favour of the concept that they represent specific chromosome segments (Lafontaine & Lord, 1969; Church & Moens, 1976).

MATERIALS AND METHODS

Roots from Allium porrum seeds germinated in damp vermiculite were used for the present study. Portions 0.5 to 1 mm in length were excised from the root tips and fixed in ice cold 1 % osmium tetroxide, 4 % formaldehyde or in 2.5 % glutaraldehyde adjusted to pH 6.8 with 0.075 M sodium cacodylate. Some specimens were also fixed with either of these aldehydes and with osmium tetroxide.

For enzymic digestion studies roots fixed in formaldehyde, as above, were treated with the following enzymes:

Deoxyribonuclease. A 0.1 % solution of this enzyme (DNase I, chromatographically purified: Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was prepared in 0.5 M Tris-HCl buffer, pH 7.0, containing 0.003 M MgSO₄. Treatment was carried out at 37 °C for 30 min to 2 h.

Ribonuclease. This enzyme (RNase I, purified by gel electrophoresis: Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was used at a concentration of 0.2 % in 0.05 M Tris-HCl, pH 6.8. Hydrolysis of the specimens was carried out at 37 °C for 1-3 h.

Proteinase K. Specimens were digested at 37 °C for 15 or 30 min with a 0.0025 % solution of this enzyme (BDH Chemicals) in 0.05 M Tris-HCl, pH 8.0. Certain specimens were first treated with ribonuclease for 1 h and then with proteinase K for 15 or 30 min.

In all such digestion experiments, control roots were left in the buffer for periods corresponding to the digestion periods.

Formaldehyde-fixed roots were usually processed for staining with 3,3′-diaminobenzidine (DAB) according to Anteunis, Pouchelet, Robineaux & Vial (1973). Some of these roots were instead stained with a 2 % ethanolic solution of phosphotungstic acid (PTA) as recommended by Sheridan & Barnett (1969). The Feulgen-like thallium ethylate staining procedure (Moyne, 1973) was carried out on ultrathin sections of glutaraldehyde-fixed roots. The latter fixative was equally used in the case of specimens stained by the uranyl-acetate-EDTA-lead citrate procedure (Bernhard, 1969).

Fig. 1. Portion of an early S nucleus containing 2 puff-like formations both of which are localized within the peripheral portion of the nucleus. It should be noted that the fibrillar structure closest to the nuclear envelope is actually associated with it by means of compact chromatin projections (arrow). Similar small, fingerlike chromatin masses are observed all along the nuclear envelope. One of the loose fibrillar formations is noticeably larger than the other and most probably represents such fused structures. Osmium tetroxide fixation and staining in 2 % aqueous uranyl acetate. x 31,000.

Fig. 2. In this mid-G₁ nucleus, the thick chromatin strands have not yet completely unraveled and form a coarse reticulum. Four fibrillar formations (arrows) are present in this portion of the nucleus. Due to the plane of sectioning 2 of these structures appear to be situated at the extremity of a chromatin strand. A third one is surrounded almost completely by dense chromatin. Owing to staining with an ethanolic solution of uranyl acetate, the dense chromatin is quite opaque and the puff-like formations exhibit a coarse chromatin meshwork. Glutaraldehyde-osmium tetroxide fixation. x 40,000.
For radioautography, roots were immersed in aerated tap water containing 10 to 20 μCi/ml of [6-3H]thymidine (sp. act. 8 to 10 Ci/mmol: Schwarz-Mann, Orangeburg, N.Y.) for 20–30 min. Following labelling, the specimens were washed in tapwater and the root tips fixed in 1 % osmium tetroxide buffered to pH 7.2 in 0.1 M sodium cacodylate. The ultrathin sections obtained from Epon-embedded blocks were affixed to collodion-coated slides and then covered with a uniform monolayer of Ilford L-4 bulk emulsion according to the technique described by Kopriwa (1967). After 2–3 months exposure, the preparations were developed by the gold latensification-Elon ascorbic acid method (Wisse & Tates, 1968). Staining of these specimens was carried out with both uranyl acetate and lead citrate solutions according to standard procedures.

RESULTS

General organization, size and distribution

The interphase nucleus in plant species, such as Allium porrum, consists of a dense chromatin reticulum, the overall organization of which varies to a noticeable extent during the G1, S, and G2 periods (Lafontaine & Lord, 1974). Throughout interphase certain segments of the dense chromatin strands exhibit a much looser texture and appear as more or less spherical structures. In material fixed with osmium tetroxide, formaldehyde, glutaraldehyde or with aldehyde-osmium tetroxide, these formations...
are seen to consist of 2 main components: (a) fine convoluted fibrils which, at sufficient magnification, can usually be verified to be continuous with the immediately adjacent segments of chromatin; and (b) a pervading, lighter substance or matrix. As is also the case for the dense chromatin strands, the former fibrillar component shows a finer texture in material fixed with osmium tetroxide (Figs. 2–4). This fibrillar meshwork appears particularly coarse in specimens which were heavily stained with an ethanolic solution of uranyl acetate (Fig. 4). Moreover, nuclei which occasionally consist of more condensed or clumped chromatin strands also invariably show spherical structures which contain a much coarser fibrillar component.

Although it is difficult to gain a clear view of the distribution of the centromeres throughout the interphase nucleus from the examination of individual ultrathin sections, one cannot help noticing that they vary noticeably in number from section to section. Many sections do not, indeed, show any of these loose spherical structures whereas others contain from 1 to 6. One or two such structures are often closely associated with the nucleolar surface (Figs. 4–6) or may occasionally also be seen close to the nuclear envelope (Fig. 1). Examination of hundreds of micrographs also reveals that, when several spherular bodies are present within a given preparation, they are not scattered throughout the nuclear cavity but are instead grouped in one area of the

Fig. 9. Micrograph showing 3 loose fibrillar formations which are grouped in portion of a late S nucleus. Following staining with thallium ethylate the chromatin strands become quite contrasty whereas the nucleoplasm shows no ultrastructural details. The puff-like formations react positively and can be clearly seen to consist of a fine fibrillar meshwork. Two chromatin strands are intimately associated with opposite sides of each of the 3 loose fibrillar formations, thus clearly illustrating the structural continuity which in fact exists between them and neighbouring portions of chromosomes. × 20,000.

Fig. 10. Micrograph of a preparation stained with 2 % uranyl acetate for 15 min and then exposed to EDTA during 30 min. The nucleolus was unaffected by this treatment and, although the chromatin strands retained part of their density, they nevertheless lost their fibrillar texture. A portion only of the puff-like mass (arrow) has been bleached by EDTA, the remaining fibrillogranular material forming a coarse reticulum. × 34,000.

Fig. 11. When the EDTA treatment is extended to 50 min the chromatin strands become almost totally transparent but their contours remain recognizable on account of the presence of dense granules throughout the nucleoplasm. The puff-like nuclear structure (arrow) has likewise become more extensively bleached thus indicating that it consists partly of chromatin. The persisting matrix constituent exhibits a fibrillogranular texture. × 34,000.

Fig. 12. After digestion of specimens with DNase for 2 h and subsequent staining with DAB for 3 h, the chromatin strands become totally extracted; their contours can be recognized by virtue of the persistence of the nucleoplasm which pervades the nuclear cavity. The puff-like formations have been only partly digested by this enzyme and they now consist of a loose meshwork of material staining almost as densely as the nucleoplasm. × 28,000.

Fig. 13. Micrograph of a specimen processed as in Fig. 12. In this case the loose fibrillar formation is intimately associated with the surface of the nucleolus. It may be noted that the texture of the persisting matrix material differs from that of both the nucleoplasm and nucleolus. × 28,000.
Cytochemistry of fibrillar nuclear structures
nucleus. The fact that much larger ellipsoidal loose fibrillar formations are also sometimes observed suggests that neighbouring similar structures have fused during previous periods of interphase (Fig. 1).

A further and most important feature of these fibrillar nuclear structures is their association with the dense chromatin reticulum which characterizes interphase nuclei in this plant species. Although a few of these sperical formations appear to lie free in the nucleoplasm (Fig. 4), most such structures are continuous with chromosome strand segments. They may be either partly surrounded by large chromatin masses, presumably chromocentres (Fig. 2), or located between 2 segments of chromatin strands (Figs. 3, 9). Stereoscopic electron micrographs of relatively thick sections easily permit one to follow the fine chromatin fibrils from the spherical structures into the neighbouring chromatin masses (author’s unpublished observations).

Cytochemical characteristics

In order to gain information on the composition of these loose spherical structures, preparations were stained according to procedures which are known either to be specific for DNA, RNA and proteins or to react preferentially with these substances. Other preparations were first digested with appropriate enzymes before staining.

DNA

Following staining according to the thallium ethylate procedure (Moyne, 1973), a technique which is preferential for DNA, the chromatin reticulum stands out conspicuously although its density is less than that observed in specimens processed with uranyl acetate and lead citrate. More transparent Feulgen-positive spherical structures (Fig. 9) are also observed throughout the nuclear cavity and, judging from their size, their loose texture and their close association with the chromatin strands, they unmistakably correspond to the fibrillar formations observed in preparations stained by conventional procedures.

In material processed according to the EDTA regressive staining technique for 30–50 min, the chromatin strands have become much more transparent than the nucleolus and now appear rather amorphous in texture. The fibrillar nuclear structures have likewise become less contrasty as a result of bleaching of part of their fibrillar meshwork (Fig. 10). After 50 min of treatment with EDTA the bleached portions of these formations closely match the neighbouring chromatin strands in density but there still persists within their mass a meshwork of stained fibrillar granular material, presumably made of ribonucleoproteins (Fig. 11).

A still more specific localization of DNA within these structures is achieved by deoxyribonuclease digestion of formalin-fixed material followed by staining with DAB. In material digested for 2 h with this enzyme, the chromatin strands do not stain at all with DAB. The fibrillar bodies under discussion now show numerous small lacunae but they maintain their puff-like organization due to the persistence of a loose reticulum. It is also evident that this persisting loose meshwork is not identical in texture and staining intensity (Figs. 12, 13) to the nucleoplasm.

Labelling experiments with tritiated thymidine were also carried out to verify
whether incorporation of this specific DNA precursor could be demonstrated within the nuclear spherical formations. Taking into account that thymidine takes approximately 5 min to reach the meristems (Evans, 1964), roots were exposed to this precursor for approximately 15 min. Shorter exposure to this precursor furnished too few radioautographic grains over the nuclei to study their labelling pattern. Longer exposure to thymidine proved equally unsatisfactory for our purpose on account of the large population of grains over the dense chromatin strands immediately adjacent to the spherical structures and of the increased difficulty of determining the exact localization of the radioautographic labelling. Under these experimental conditions, no labelling was observed over the loose spherical formations in early S nuclei and approximately one third of these puff-like structures showed radioautographic grains in mid-S nuclei (Figs. 7, 8). Although some of the silver grains were often localized over the central portion of these structures, in all cases the labelling was noticeably higher at their periphery. In view of the close association of dense chromatin strands, which are also labelled in such nuclei, with the centromeres it is most likely that some of the silver grains originate from the neighbouring dense chromatin masses.

**RNA**

As described above, bleaching with EDTA of uranyl acetate-stained preparations leads to a progressive decrease of the overall density of the fibrillar bodies (Figs. 10, 11). Following a 50-min EDTA treatment their chromatin meshwork is no longer visible and there only persists a rather coarse fibrillogranular component the density of which closely matches that of the nucleolar mass as well as that of the fine fibrils and granules scattered throughout the nucleoplasm or bordering the chromatin strands.

The presence of RNA within centromeres was demonstrated more conclusively, perhaps, by digesting roots for 2 h with ribonuclease and subsequently staining them with DAB. Under these experimental conditions the staining reaction is specific for RNA (Roels & Goldfischer, 1972; Anteunis et al. 1973). Following such treatment, the cytoplasmic ribosomes and the nucleolar particulate component are disorganized. Although the overall fibrillar architecture of the puff-like formations is maintained, it is nevertheless evident from their greater transparency that part of their mass has been extracted by this enzyme (Fig. 14).

**Proteins**

For these studies, blocks of tissue were either stained with PTA following glutaraldehyde fixation or were digested with proteinase K following preservation with formaldehyde.

In material stained with ethanolic PTA, the dense chromatin strands as well as the loose structures (Fig. 15) show a less distinctly fibrillar texture than in specimens processed according to the DAB staining procedure (Figs. 5, 6). At sufficient magnification, the impression is gained that these puff-like formations consist of a slightly denser particulate component embedded in a pervading, amorphous substance. When tissues are digested with proteinase K for 30 min and stained with DAB, the interphase chromatin strands still exhibit a fibrillar texture and their compactness is only
slightly less than in untreated material. The fibrillar meshwork characterizing the spherical formations appears, however, slightly looser and less opaque (Fig. 16) than in control specimens as a result of the apparent extraction of part of the pervading matrix material. A larger portion of this matrix is removed following digestion of specimens with both RNase (1 h) and proteinase K (15 min) in this order (Fig. 17). When this double digestion involves a longer proteinase K treatment (30 min), the matrix portion becomes totally transparent (Fig. 18). Certain nuclei are noticeably more extracted by this latter treatment and thus exhibit spherical structures (Fig. 19) consisting uniquely of a rather coarse fibrillar meshwork which, in such cases, is clearly seen to be continuous with similar fibrils emerging from adjacent segments of chromosomes.

**DISCUSSION**

Nuclear structures similar in organization to those described in this report have, over the last few years, been observed in different plant species and were referred to as karyosomes (Falk, 1962; Sankaranarayanan & Hyde, 1965; Hyde, 1967), micropuffs (Lafontaine & Lord, 1969; Risueño et al. 1978) and centromeres (Church & Moens, 1976; Moens & Church, 1977). Two main hypotheses have emerged from these various studies as to the nature of these structures. According to certain authors, these nuclear formations correspond to inclusions (Risueño et al. 1978) whereas other investigators believe that they represent specific segments of chromosomes (Lafontaine, 1974a, b; Church & Moens, 1976; Moens & Church, 1977).

Two types of observations have been invoked by Risueño et al. (1978) in favour of the notion that these loose fibrillar structures are not chromosomal in origin but are

---

**Fig. 14.** Portion of a nucleus from specimen digested with RNase for 2 h and subsequently stained with DAB. The chromatin strands (arrow) were little affected by this treatment whereas the matrix of the puff-like formations was partly extracted. The protein component of this matrix is not stained by this technique with the consequence that it appears quite transparent. × 48000.

**Fig. 15.** When specimens are stained with ethanolic PTA, the chromatin strands become quite dense. The loose spherical structures also appear relatively contrasty and take on a more granular texture than in preparations stained with uranyl acetate-lead citrate or with DAB. × 48000.

**Fig. 16.** After digestion of specimens with proteinase K for 30 min and staining with DAB, the ultrastructure of the puff-like formations resembles very closely that observed in RNase-treated material (Fig. 14). In view of the preferential staining of both DNA and RNA by DAB, this result indicates that a sizeable portion of their mass consists of these 2 substances. × 40000.

**Figs. 17-19.** These 3 figures illustrate the effect on the loose fibrillar formations of 1 h digestion with RNase followed by proteinase K for 15 min (Fig. 17) or 30 min (Figs. 18, 19). As already suggested by Figs. 14 and 16 considered jointly, extraction of RNP material from these structures does not disrupt their puff-like architecture. The persisting DAB-stained meshwork must therefore consist of DNA. In cases where these nuclear formations have undergone more extensive extraction (Fig. 19), the continuity between their DNA meshwork and the neighbouring chromosome strands is particularly easy to recognize. × 49000, 44000 and 41000, respectively.
rather somehow related to the nucleolus. Firstly, it is argued that a few of these bodies are sometimes closely associated with the nucleolar surface and that they also consistently possess the same cytochemical characteristics as the nucleolar components. Earlier observations with *Allium cepa* (Lafontaine, 1965) as well as the present ones with *Allium porrum* reveal that, whatever their localization within the nucleus, these structures are often seen, under both light or electron microscopy, to be continuous with immediately adjacent chromatin strands. As for their cytochemical characteristics, we agree with these authors that the nuclear spherical structures react rather similarly to the nucleolus with certain cytochemical tests. In our view, this is to be expected since both nucleolus and these puff-like formations not only contain ribonucleo-protein material but chromatin as well. A second type of argument brought forth by Risueno *et al.* (1978) to document their view as to the non-chromosomal nature of the micropuffs rests on the observation that the number of these nuclear formations varies during interphase and, moreover, that their average number is greater than the number of chromosomes. It is to be noted, however, that these data have been obtained by counting bodies reacting with silver in squashes of roots treated with 0.1% caffeine for 1 h. This drug is unfortunately known to induce the formation of fibrillar spherical bodies, in plant interphase nuclei, the ultrastructure of which is different from the puff-like structures under discussion (Moreno Diaz de la Espina & Risueno, 1977). Data obtained from normal cells would therefore be more appropriate.

The original hypothesis (Lafontaine & Lord, 1969) that these nuclear structures are micropuffs and therefore correspond to relaxed segments of chromosomes rested mostly on the following observations: (a) In *Allium cepa*, such formations are usually continuous with neighbouring chromatin strands and consist predominantly of similar fine fibrillar material; (b) These distended chromosome regions also stain metachromatically with azure B. Later ultrastructural studies (Colman, Stockert, Esponda & Risueno, 1972; Dontigny, 1973), using Bernhard's (1969) staining technique confirmed the presence of RNP within these nuclear structures and thus added further support to the above concept.

The present cytochemical data conclusively prove, we believe, that the nuclear structures under discussion consist basically of a loose meshwork of chromatin fibrils and of a pervading RNP matrix. These structures, indeed, react positively with thallium ethylate, a Feulgen-like stain which has been shown to be preferential for DNA (Moyne, 1973). Likewise, our results show that the fine fibrillar meshwork, revealed in these structures by conventional stains, is bleached when preparations are exposed to EDTA according to Bernhard's (1969) procedure. Digestion experiments with deoxyribonuclease of formalin-fixed specimens furnish still more conclusive data in support of the presence of DNA within these nuclear formations. It should be pointed out that our attempts to use deoxyribonuclease on glutaraldehyde-fixed roots were unsuccessful in accord with previous studies using the same experimental conditions (discussed in Bouteille, Laval & Dupuy-Coin, 1974). Another piece of evidence pointing to the presence of DNA within the fibrillar structures comes from the observation that they are labelled with tritiated thymidine. Although the labelling pattern observed is quite suggestive, the possibility must nevertheless be envisaged.
that some of the silver grains lying over these loose formations originate from the immediately adjacent segments of the chromatin strands which, in these mid-S nuclei, are still relatively active in DNA synthesis (Lafontaine & Lord, 1974). This problem is now being investigated using the higher resolution radioautographic technique recently described by Bouteille (1976).

The examination of serial sections of both root tip and premeiotic interphase nuclei (Allium fistulosum) has recently furnished much new interesting data on the distribution, number and variation in size of these puff-like fibrillar formations (Church & Moens, 1976; Moens & Church, 1977). These studies have shown, in particular, that the number of these fibrillar formations per nucleus is less than the diploid number of chromosomes, presumably as a result of fusion. On account of their typical polar clustering and of the fact that multiple centromeres are associated with centric heterochromatin masses, these nuclear formations were assumed to be centromeres. Preliminary observations of serial sections of Allium porrum interphase nuclei are consistent with this view. At early G1, for instance, when the chromosomes are still well aligned within the forming nucleus (Lafontaine & Lord, 1974), several puff-like formations may be observed throughout the polar portion of the nuclear cavity. Such distribution is highly suggestive that these structures indeed correspond to the chromosome centromeric regions.

Although the present report shows that the loose fibrillar structures observed in Allium porrum contain DNA, it should be pointed out, for purpose of clarification, that this is not the case of the spherical nuclear formations in certain other plant species. In plants, for instance, with relatively low DNA content and chromocentric interphase nuclei (Raphanus sativus), one or two globular bodies are often closely associated with the nucleolar surface. These have usually been referred to as karyosomes (Sankaranayanan & Hyde, 1965; Hyde, 1967), spherules (Lafontaine, 1965), micronucleoli (Lafontaine, 1968) or loose nuclear bodies (Jordan, 1976). Recent cytochemical studies of these structures in a few plant species with chromocentric interphase nuclei strongly suggest that they do not contain DNA and exhibit an ultrastructural organization different from that of the puff-like formations described in this report (authors' unpublished observations). Such structures undoubtedly do not correspond, therefore, to centromeres and their nature remains obscure.

It is a pleasure to thank Dr A. Lord, who participated in the early phase of this work as well as Dr M. Pouchelet for his most useful advice. The authors also gratefully acknowledge the excellent technical assistance of Mrs Diane Michaud and Mr Siegfried Gugg. This investigation was supported by research grants from the Ministry of Education of Quebec and the National Research Council of Canada.

REFERENCES


(Received 22 November 1978 – Revised 22 March 1979)