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Ultrastructure of the Sex Chromosomes and Nucleolus in Early Spermatids of *Marmosa elegans* and *Dromiciops australis* (Didelphoidea - Marsupialia)*

Ultraestructura de los cromosomas sexuales y del nucléolo de las espermátidas tempranas de *Marmosa elegans y Dromiciops australis* (Didelphoidea – Marsupialia)

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The recent finding of nucleolar activity at the initial stages of spermiogenesis and the presence of Feulgen–positive chromocenters of constant size and position in the nucleus of early spermatids (ES) from *Marmosa elegans* and *Dromiciops australis* led us to study the chromatin and nucleolus organization in the ES of these species.

The seminiferous tubules were fixed in 2.5% glutaraldehyde stained with ethanolic PTA, or post-fixed in 1% OsO_4. Observations were also made in material stained with PAS hematoxylin or Feulgen. Somatic chromosomes were stained with the AgAS-NOR technique.

Three stages were defined for ES following meiosis I and II: ES-1 with a telophasic nucleus, ES-2 with spheric nucleus and ES-3 with a slightly ovoid nucleus and an incipient acrosomic groove. ES-2 and ES-3 bear a conspicuous chromocenterr (1.5 to 2μm) irregularly ovoid and adhered to the inner layer of the nuclear envelope, or a small one (0.5 to 0.6μm) in a central position close to the nucleolus. Both chromocenters are similar in structure and cytochemical preference (PTA) to the sexual bivalent of primary spermatocytes. ES bear central nucleoli whose number is equal to that of the nucleolar chromosomic pairs of each species. The large ES-2 nucleolus is formed by concentric trabecules, of an essentially fibrillar material. In ES-3 the nucleolus is located in the periphery and its components are segregated, allowing to clearly distinguish the NOR.

The structural and cytochemical characteristics of the chromocenters that have been identified and the ultrastructure of their chromatin allow to propose that they correspond to the X and Y chromosomes. The morphology and structure of the nucleolus reveals nucleolar activity and its subsequent involution.

In studies of mammal spermiogenesis, special attention has been devoted to changes in shape and condensation of the spermatid nucleus in intermediate and advanced stages, to the development of the acrosome, and to the growth and differentiation of the flagelum. In contrast, less attention has been given to the nucleus and

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chromatin in early spermatids. From birth until the initial stages of acrosome formation the nucleus of early spermatids attains interphasic features with the organization and functionality of an haploid genoma with one or several nucleoli. Data in the literature in relation to these facts are scanty and rather indirect.

Daoust and Clermont (1), when studying the distribution of nucleic acids in the different cell types of the rat testis, reported the presence of nucleoli and chromocenters in early spermatids. Subsequently, Monesi (2) based on studies of incorporation of ^{3}HUdr into the mouse testis, communicated the existence of a small but significant RNA synthesis during the early stages of spermiogenesis. On the other hand, Kiersenbaum and Tres (3) observed that their radioautographic studies in spermiogenesis do not support a nucleolar organization in the early spermatids of the mouse. In turn, Yasuzumi (4) has communicated the existence of prominent nucleoli in early spermatids of some vertebrate species, although he remarks though that in many animal species the appearance of nucleoli in spermatids is not clearly understood.

Recently, Berríos and Fernández-Donoso (5-6) have reported the existence of nucleoli and chromocenters of constant size and position in the nuclei of early spermatids of two marsupial species. Moreover, Krimer and Esponda (7) refer to the presence of nucleoli in young spermatids of the mouse. On the other hand Hofgärtner *et al.* (8), by using the AgAS - NOR technique have described the presence of active nucleolus organizer regions (NOR) in the nuclei of early spermatids of four mammals species, as well as their association to certain particular chromocenters in man and mouse spermatids.

In this work, we describe the ultrastructure and morphological organization of nucleoli and chromocenters found in early spermatids from the end of meiosis up to the appearance of the acrosomic groove, in *Marmosa elegans* and *Dromiciops australis* (Didelphoidea - Marsupialia).

MATERIAL AND METHODS

Adult males of *Marmosa elegans* and *Dromiciops australis* were captured respectively in Cachagua (Province of Aconcagua) and in the Valdivian Forest (Province of Valdivia), Chile. Their furs and skulls were deposited in the collection of the Cytogenetics Unit, Department of Cell Biology and Genetics, University of Chile.

Pieces of testes 1-2 mm in diameter were fixed in 2.5% glutaraldehyde in phosphate buffer pH 7.2 and postfixed in 1% OsO_4 in the same buffer. Part of the glutaraldehyde-fixed material was treated by the ethanolic PTA technique and embedded in Durcupan ACM (araldite). Both random and serial sections were cut on a Sorvall MT2b ultramicrotome. Sections of the material post-fixed in OsO_4 were stained with uranyl acetate alone or double stained with uranyl acetate and lead citrate. The spermatids nuclei were examined and photographed either with a Siemens Elmiscop I or a Philips 300 Electron Microscope.

Three-dimensional reconstructions were performed starting from serial sections of whole nuclei or from parts of nuclei, according to Wettstein and Sotelo (10).

Some pieces of testes were fixed in Bouin and Carnoy, embedded in paraffin and stained, either with periodic acid schiff: (P.A.S.) – Hematoxylin, to visualize acrosomal evolution in order to detect spermatogenesis stages, or with Feulgen for disclosing nuclear chromocenters.

Metaphase plates were prepared from bone marrow, and stained by the Ag-AS technique of Goodpasture and Bloom (11).

Early spermatids were named according to Rattner (12) since his classification based on the morphological changes of spermatids nuclei from *Marmosa mitis* and *Didelphis virginiana,* is deemed valid for most marsupial species (13).

RESULTS

Based upon the joint evaluation of sections, studied under light and electron microscope, and taking into account the characteristics of chromatin condensation as well as the nucleolus evolution, three

sub-stages of early spermatids (ES) have been defined: ES-1, resulting from the second meiotic division, presents a telophasic nucleus, chromatin in decondensation, and formation of the nuclear envelope (Fig. 2); ES-2 with a spheric nucleus, granular chromatin, chromocenters of constant size and position and prominent central nucleolus (Figs. 1, 4, 5 a, 5 b, 5 d, 5 e, 10); and ES-3, of slightly oval nucleus incipient acrosomic groove, finely granular chromatin, chromocenters of constant size and position, and peripheral segregated nucleolus (Figs. 6 and 7). All three evolutive stages of the ES's are sequential. Frequently an association is observed between ES-1 and ES-2 with meiotic divisions I and II, or of ES-1, ES-2 and ES-3 in the absence of meiotic divisions (Figs. 1 y 2). The ES's thus defined, are very similar in the two species studied, therefore it was decided to utilize the material of *M. elegans* as the standard for description.

a) *Chromocenters*

Using the Feulgen technique, an ES can be differentiated into two types: a) those with a Feulgen positive chromocenter attached to the nuclear envelope, and b) those where it is not possible to recognize any chromocenter amongst the granulation of the chromatin. These two types of ES are found in one some seminiferous tubule in a proportion of approximately 1 : 1. This fact becomes evident in the ES-2 (Fig. 1).

In electron microscopy sections, specially when PTA-stained, it can be seen that ES-2 and ES-3 bear either a large chromocenter, 1.5 to 2μm irregularly ovoid and attached to the inner layer of the nuclear envelope (Figs. 5 a-c) or a small central chromocenter approximately one third the size of the former (Figs. 5 d-f). These chromocenters resemble each other in their ultrastructure and in the preferential cytochemical properties of the chromatin that conforms them. In both species studied, they are also similar in structure to the sex bivalent of primary spermatocytes at pachythene (Figs. 8 a and 8 b).

b) *Nucleolus*

No nucleolus was found in the ES-1 when observed under the electron microscope. However as telophase of the II meiosis wanes and chromatin becomes decondensed, central nucleoli begin to organize in the ES-2: one in *M. elegans* (Fig. 5) and two in *D. australis* (Fig. 10). This is in keeping with the number of nucleolar chromosomic pairs of either species (Figs. 9 a and 9 b). In *Dromiciops* a difference in size is often observed between both nucleoli. In the ES-2 of both species the nucleolus is formed by a series of concentric trabecules of a fibrino-granular material. In certain sections small patches of the NOR chromatin are even distinguishable.

In the ES-3 the nucleolus, or nucleoli, are located at the periphery of the nucleus (Fig. 6) on the pole opposite to the still incipient acrosomic groove. The progressive segregation of the nucleolar components allows to clearly detect the NOR chromatin (Figs. 6 and 7).

DISCUSSION

The morphologic development of the nucleus in spermatids of *Marmosa elegans* and *Dromiciops australis* is not significantly different from that described by Rattner (12) for *M. mitis* and *D. virginiana*. The spheric nucleus of early spermatids gradually turns into a flat U-shaped nucleus, positioned at right angles to the long axis of the late spermatid (see Fig. 2). These morphological changes are directly correlated with the progressive condensation of chromatin in the nucleus. Throughout this process, the different types of spermatids closely resemble those described for eutherian mammals both in morphological features and in their duration in time (14). The presence in the same tubule of ES-1 and ES-2 with meiotic divisions, or of all three types of ES simultaneously, suggests that these substages are short-lasting. On the other hand this is further supported by the association of ES with spermatocytes during zygotene and early pachytene stages, which are

usually brief and correspond to the new wave of cells that have entered the meiotic prophase (15) (see Figs. 1 and 2).

Chromocenters:

The approximate 1 : 1 ratio of the chromocenters in ES-2 and ES-3 along with their morphological and cytochemical features, and the structure of the chromatin (Figs. 9 a and 8 b) allow to suggest that they are in fact the X and Y chromosomes segregated and distributed after the meiotic divisions.

In both species studied, the Y chromosome is dot-like (16-17). It is not visible in Feulgen stained preparations, being at the limit of resolution of the light microscope. The Y chromocenter as viewed on the electron microscope is 0.5 to 0.6 μm in size, it occupies a central position and is associated to the nucleolus. Hofgärtner *et al.* (8) found a similar position of the Y chromosome in man and mouse. In both cases the fluorescent Y chromosome appears to be intimately related to the NOR.

On the other hand, one of the stronger arguments allowing to postulate that these chromocenters are the sex chromosomes, is the similarity of their chromatin ultrastructure to that of the sex bivalent during the meiotic prophase. This is important since it points to an upkeeping of a specific condensation for sex chromosomes along meiosis and spermiogenesis (17, 18). Such features are more evident in the X chromosome which is more prominent and adheres to the inner surface of the nuclear envelope. The subsequent chromatin condensation in middle and late spermatids does not allow to detect in these cells a differential condensation –if any– in sex chromosomes. Notwithstanding, we believe that this peculiar behaviour of the chromatin of sex chromosomes –specially X chromosome– lends some structural and morphological support to the hypothesis of preferential inactivation of paternal X in marsupial females. This condition might be acquired during male meiosis (19-20).

Nucleoli

In studies on the incorporation of radioactive precursors of RNA synthesis into early spermatids of the mouse, Monesi and other workers (21-23) detected a certain amount of RNA production which was subsequently characterized as heterogeneous nuclear RNA (23, 24). Besides Geremía *et al.* (25) found that an important percentage of the RNA species synthesized in such spermatids, corresponded to ribosomal RNA. These works also show that the synthesis of RNA's of any kind gradually disappear as differentiation progresses (23). In turn Hofgärtner *et al.* (8) based upon their studies of spermatogenesis in 4 mammal species (including man), carried out by the ammoniacal silver technique, have shown the existence of a postmeiotic activity of the NOR in early spermatids. This activity, inferred from the presence of Ag NOR positive zones in the the spermatids, slowly decreases till waning during the more advanced stages of spermiogenesis.

Our observations in early spermatids of *M. elegans* and *D. australis* show the appearance of nucleoli and their subsequent segregation (5). We know that the nucleolus corresponds to the region of the nucleus where ribosomal RNA is synthesized and stored, and also that roughly speaking nucleolar segregation to an involution of such synthesis (26). Therefore our data are in agree with those of Monesi *et al.* (23) and Hofgärtner *et al.* (8), strongly suggesting that the NOR's after meiosis, reassume their synthesizing function which gradually decreases thereafter. Moreover the presence of one nucleolus in *M. elegans* and of two nucleoli in *D. australis* suggests that in early spermatids of these species the NOR's are active and maintain their structural and functional individuality (6). This phenomenon could have arisen in the meiotic prophase during the condensation of NOR chromatin (27).

Summarizing, the recent identification of different species of ribosomal RNA in early spermatids, the positive reaction of the NOR's of spermatids to ammoniacal

silver after negative reaction during meiotic divisions, as well as the actual presence of nucleoli, point to the existence of a true nucleolar activity in this stage of spermiogenesis.

The paucity of information available makes it difficult to interpret the actual meaning of the activity of NOR chromatin in the haploid nucleus.

Further studies are needed to inquire into the fate of such nucleolar material. Observations should be made in a greater number of species as to be able to generalize its occurence.

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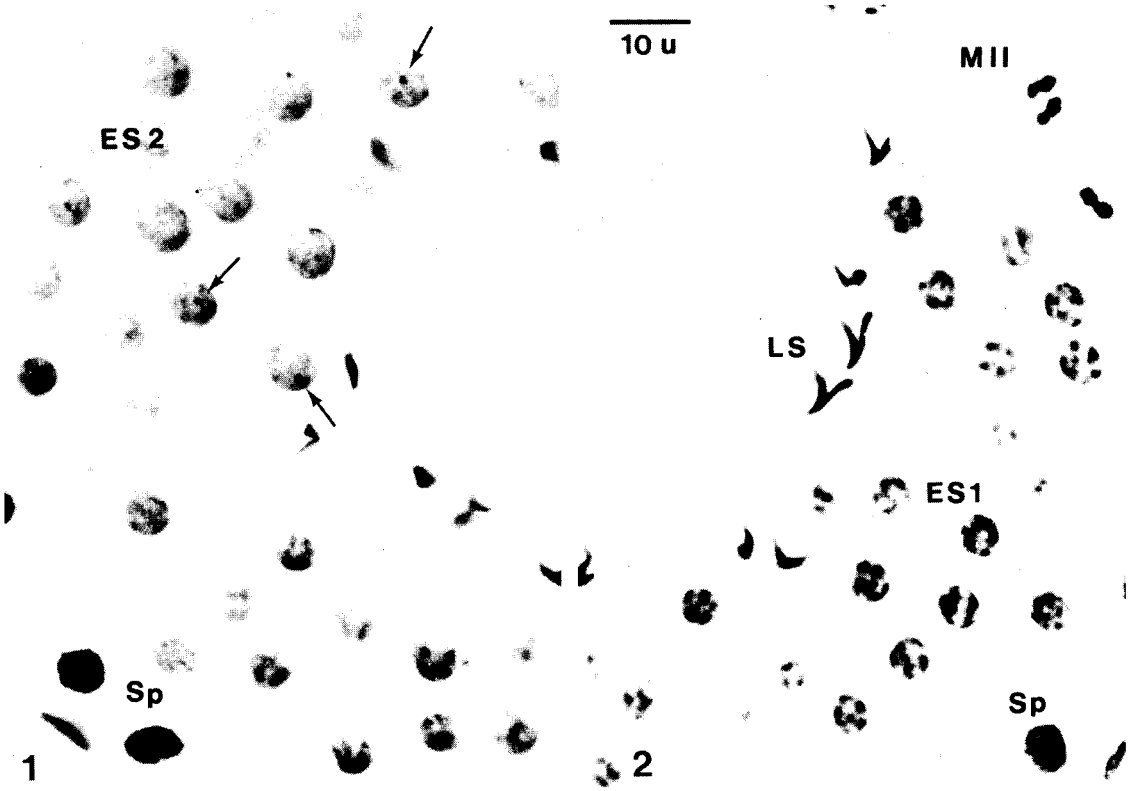

Fig. 1: Section of a seminiferous tubule from *M. elegans* sp: spermatocytes in Zygotene; ES-2: early spermatid 2 with and without peripheral chromocenter. Feulgen stain.

Fig. 2: Another sector of the same section. sp: spermatocyte in Zygotene; M II: Meiosis II; ES-1: early spermatids 1. LS: late spermatids (see text). Feulgen stain.

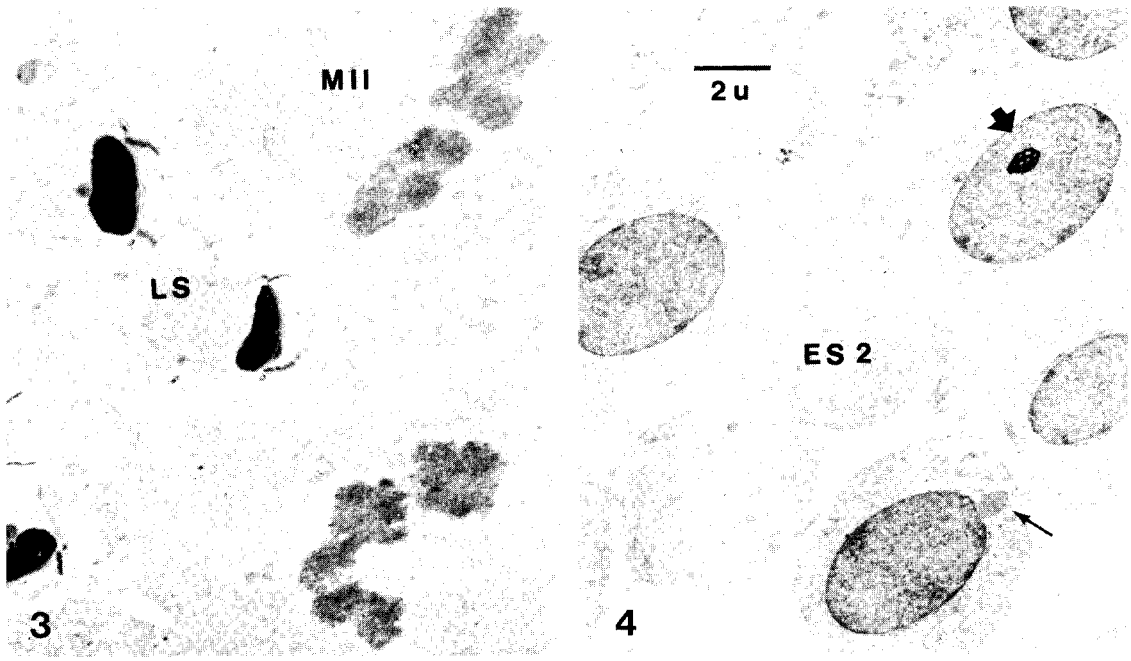

Fig. 3: Section of a seminiferous tubule from *D. australis:* M II: meiosis II. LS: late spermatids. PTA stain.

Fig. 4: Another sector of the same tubule as in Fig. 3 ES-2: early spermatids type 2. Arrow: nucleolus; small arrow: proacrosomal granule. PTA stain.

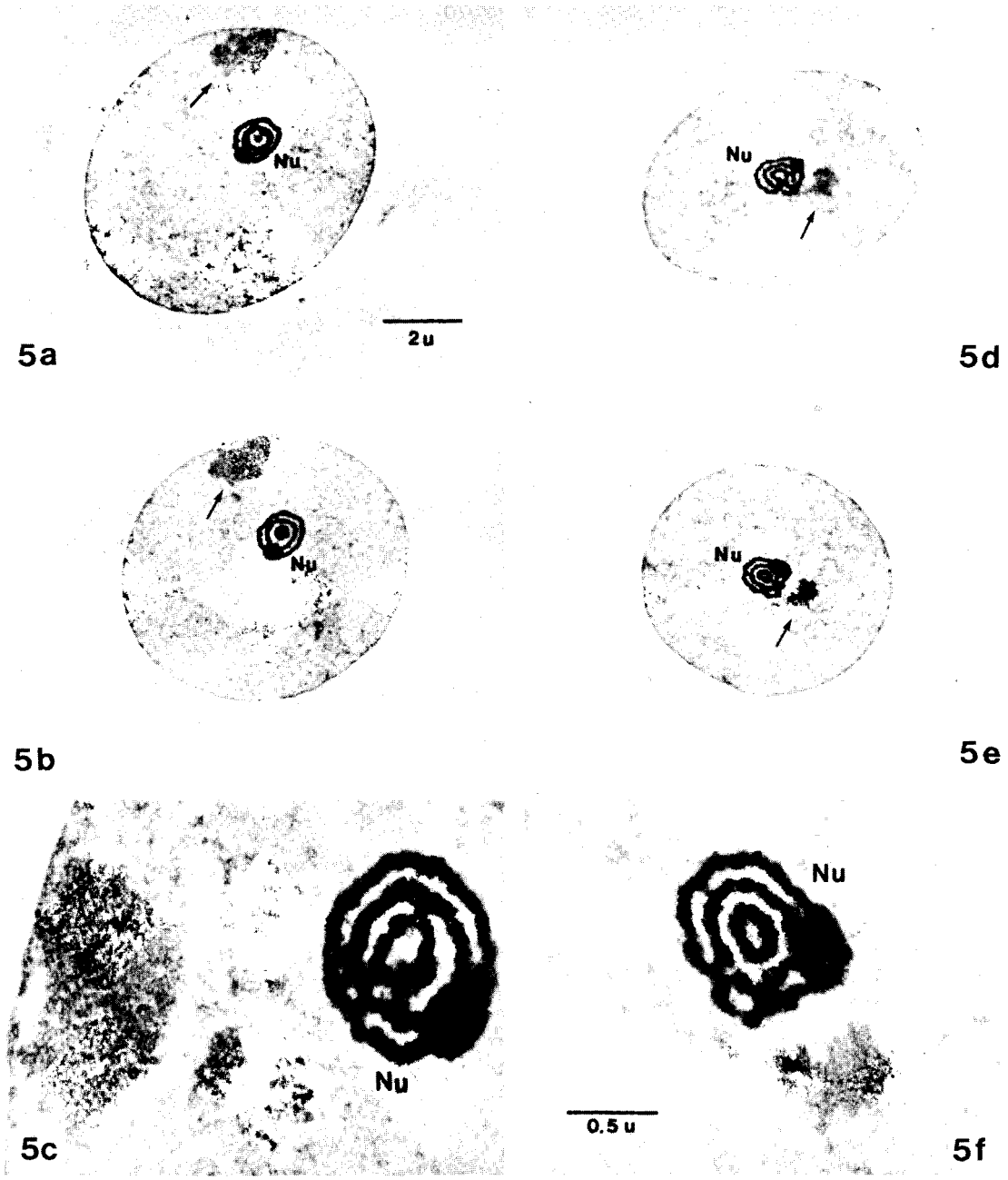

Fig. 5: (a) and (b): sections of a series of ES-2 nucleus from *M. elegans*. Arrow: round nucleus with peripherally located chromocenter; Nu: central nucleolus. PTA stain. (c): Higher magnification of the same series. The chromocenter is attached to the nuclear envelope. Nu: nucleolus (mostly fibrillar). PTA stain. (d) and (e): Arrow: round nucleus with central chromocenter and nucleolus (Nu). PTA stain. (f): Higher magnification showing the small chromocenter and the nucleolus (Nu). PTA stain.

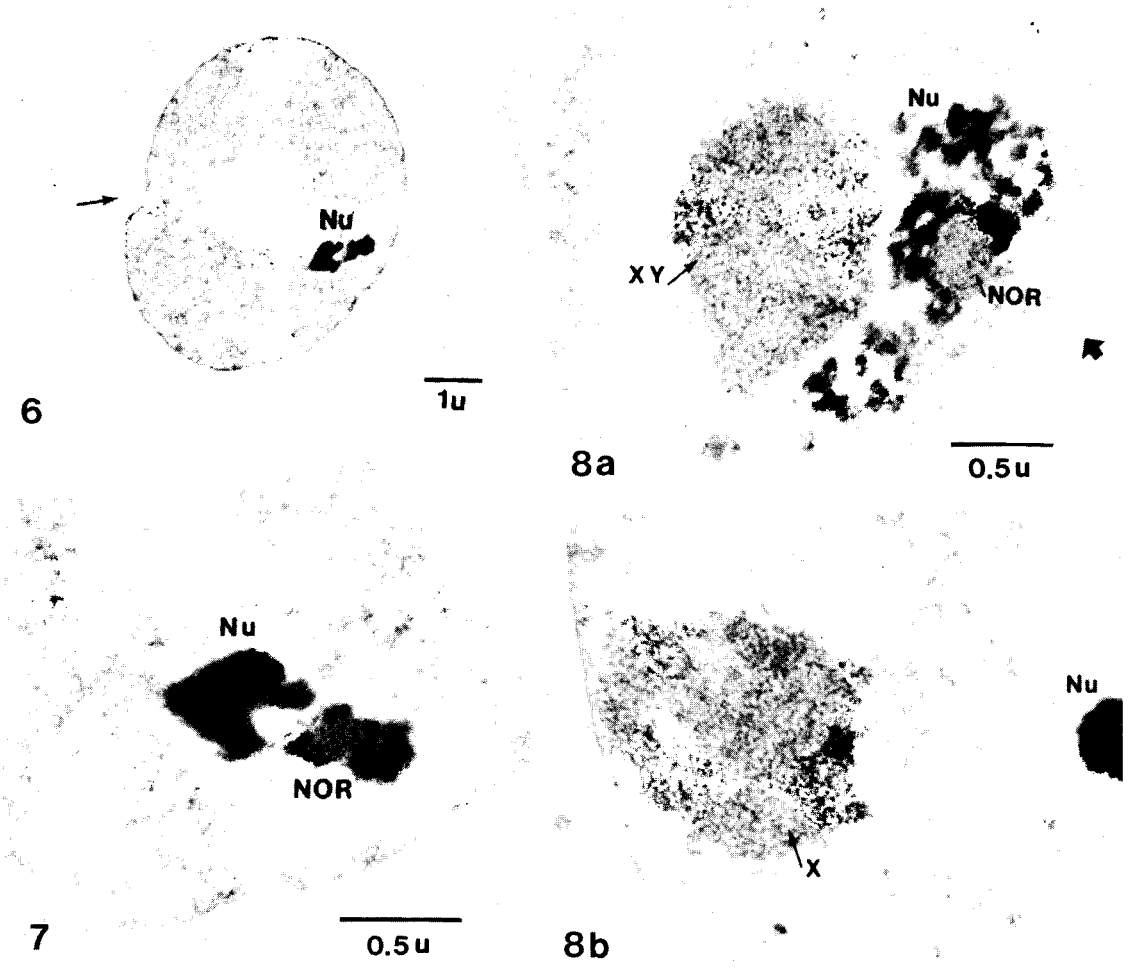

Fig. 6: ES-3: early spermatids type 3 of *M. elegans.* Arrow: ovoid nucleus with small acrosomal groove. Nucleolus peripherically located in the opposite side with respect the acrosomal groove.

Fig. 7: Higher magnification of the segregated nucleolus shown in Fig. 6. The NOR is clearly visible, as well as two masses of nucleolar material (Nu). Granules scarce or absent. PTA stain.

Fig. 8: (a): Higher magnification of a spermatocyte of *M. elegans.*
XY: sex bivalent; Nu: nucleolar material. NOR: nucleolar organizer region. Arrow: nuclear envelope. PTA stain.
(b): Higher magnification of an ES-2 from *M. elegans.*
X: large chromocenter attached to the nuclear envelope assumed to be the X chromosome (see text) Nu: nucleolar material. PTA stain.

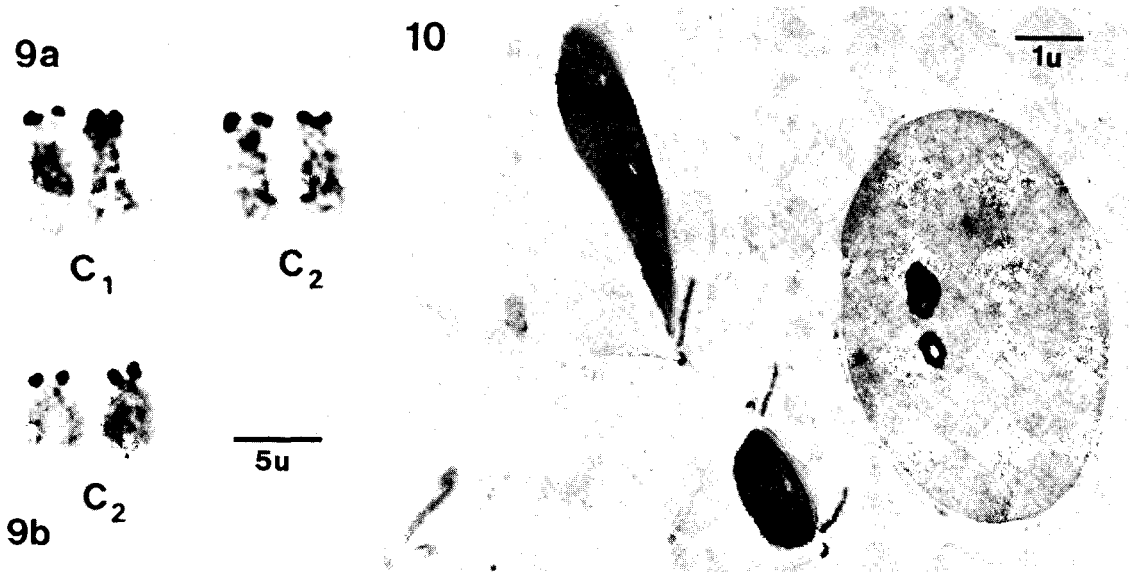

Fig. 9: Nucleolar chromosomes with the AgAS-NOR reaction.
(a): C_1 and C_2 pairs from *D. australis;*
(b): C_2 pair from *M. elegans.*

Fig. 10: Section of an ES-2 with two nucleoli from *D. australis.* Uranyl lead stain.