

Immunocytochemical localization of histones H2B, H3 and H4 in pronuclei and four-cell stages of porcine embryos. Preliminary results

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Summary — In this preliminary work, using pig embryos ultrastructural immunocytochemistry with polyclonal antibodies against purified histones was used to demonstrate both their localization and the time of their appearance in pronuclei, from 15 h after ovulation (pronuclear stage) to 48 h postinsemination (4-cell stage).

In pronuclei, the histones H2B, H3, and H4 were located in the heterochromatin as soon as it appeared. Usually, one of the pronuclei seemed to be more heavily labelled. The chromatin facing the zone of pronuclear contact formed a bowl-shaped region in each pronucleus heavily labelled for these histones. The so-called pseudo-nucleoli were present in both pronuclei and contained H2B.

In 4-cell stages, the labelling intensities of heterochromatin for H2B, H3 and H4 were equal in all the nuclei. H2B was still evident in the pseudo-nucleoli, but in a lower quantity than before. The condensed chromatin located either under the nuclear envelope or surrounding the pseudo-nucleoli was heavily labelled for H2B, H3 and H4.

fertilization — nuclei — histone — ultrastructural immunocytochemistry — pig embryo

Résumé — Localisation immunocytochimique des histones H2B, H3 et H4 dans les pronuclei et au stade quatre cellules des embryons de porc. Résultats préliminaires. *Dans ce travail préliminaire, l'immunocytochimie ultrastructurale utilisant des anticorps polyclonaux purifiés a permis de localiser les histones H2B, H3 et H4, et de dater leur mise en place dans les noyaux d'œufs âgés de 15 h (pronuclei) et d'embryons porcins à 48 h (4 cellules) après l'ovulation.*

Dans les pronuclei, les histones H2B, H3 et H4 sont associées à l'hétérochromatine dès sa formation, un pronucleus étant habituellement plus marqué. Dans chacun des pronuclei, le marquage de la chromatine périphérique est particulièrement important sous les portions d'enveloppes nucléaires qui entrent en contact avec l'autre pronucleus. Les pseudo-nucléoles des deux pronuclei lient spécifiquement les anticorps anti-H2B.

Dans l'embryon de 4 cellules, les histones H2B, H3 et H4 sont présentes dans l'hétérochromatine. Celle-ci est marquée plus intensément à la périphérie des noyaux, sous l'enveloppe nucléaire, et autour des pseudo-nucléoles, l'intensité du marquage étant équivalente dans chaque noyau. H2B est présente en quantités moindres dans les pseudo-nucléoles.

fécondation — nuclei — histones — immunocytochimie ultrastructurale — embryon porc

INTRODUCTION

Protamines, spermatozoon-specific nucleoproteins, replace histones in spermatid nuclei (Courtens *et al.*, 1983, 1988). They are rapidly eliminated from the nucleus of the male gamete soon after fertilization (Kopečný & Pavlok, 1975), leaving bare "male" DNA. In early embryo development, the DNA is reassociated with new nucleoproteins that are synthesized *de novo* or are already present in the oocytes. Among them, the histones are produced early after fertilization and their amounts correspond to the amounts of replicated DNA that are necessary for cellular division (Kaye & Wales, 1981). In the mouse, the S phase (DNA replication) that begins 11 h after insemination lasts for about 7 h (Howlett & Bolton, 1985).

The histones are involved in the reformation of the secondary structure of the DNA, being associated to form the basal units of chromatin, the nucleosomes, formed by octamers of the 4 histones H2A, H2B, H3, and H4 (Pederson *et al.*, 1986) surrounded by two DNA spirals. Successive nucleosomes are both separated and linked together by one molecule of histone H1, also located on the DNA fiber. Active transcription is associated with H1-nucleosomes—DNA complexes (Banchev *et al.*, 1988). Non-histone nucleoproteins are also involved in the spatial organization of the chromatin (Fredericq, 1976; Bradbury, 1979; Kaye & Wales, 1981; Poccia, 1986; Homberger & Koller, 1988), but their occurrence in young embryos is far from being completely understood.

The decondensation of the human spermatozoon nucleus takes place between 1 and 6—8 h after the *in vitro* mixing of gametes (Tesařík & Kopečný, 1988), while it starts after 5 h in the sheep (Crozet, 1988). In this preliminary article,

the presence and location of histones H2B, H3, and H4 in porcine pronuclei have been studied during the S phase, *i.e.* 15 h after ovulation/fertilization, using antibodies against purified histones H2B, H3, and H4. The results are compared with those on 4-cell embryos. This stage was chosen on the assumption that the activation of genome and protein synthesis had started in the species under study (Tománek *et al.*, 1986).

MATERIAL AND METHODS

Young gilts at oestrus were inseminated 24 and 12 h before expected ovulation. Eggs and young embryos were obtained by perfusion of the oviducts at slaughter with PBS (37 °C), 17 and 48 h after ovulation. They were fixed with different fixatives that gave good morphological details but which were ineffective for immunocytochemistry. In that regard, the following technique proved the most efficient. Eggs were fixed with 1% Formaldehyde in 0.1 M phosphate buffer for 1—2 h at 4 °C. The free aldehyde groups of the fixative were blocked for 1 h in 0.5 M ammonium chloride at 20 °C (Fakan, personal communication), and the cells were dehydrated and embedded in Lowicryl K4M according to Altman *et al.* (1984). The final polymerization of blocks in gelatin capsules was obtained by soft UV irradiation (320 nm, 5W, 15 cm) for 13—16 h at 4 °C. The ultrathin sections, obtained with a Reichert ultracut, mounted on gold bare grids and processed for immunocytochemistry, were observed without counterstain under a CM 10 Philips electron microscope operated at 60 kV.

Labelling of sections

Reagents

Rabbit specific anti-histone sera had been previously tested: H2B (Muller *et al.*, 1985), H3 (Muller *et al.*, 1982), and H4 (Muller *et al.*,

1987). The anti-rabbit IgG antibodies were : goat anti-rabbit IgG (GAR) immunocoupled to peroxidase (from Nordic Immunology); donkey biotinylated anti-rabbit IgG and 15 nm colloidal gold—streptavidin coated particles (from Amersham). Buffer A was composed of PBS pH 7.2, 0.01% Tween, and 10% decomplexed goat serum. Buffer B for washing was composed of PBS pH 7.2 and 0.01% Tween.

Peroxidase method

Sections pre-incubated in buffer A for 20 min at 20 °C were treated for 16 h at 4 °C with specific antisera diluted 1/500 in the same buffer. Following extensive washing in buffer B, they were incubated for 1 h at 20 °C in 1/50 GAR/IgG coupled to horseradish peroxidase in buffer A. After several washes in PBS, the peroxidase was revealed by 3 min incubation in 22 mg diaminobenzidine + 175 ml PBS + 3 µl hydrogen peroxide, followed by extensive washing in PBS and bidistilled water and drying of the sections. The final peroxidase—diaminobenzidine products were osmicated for 45 min at 60 °C in osmium tetroxide vapor (Courtens *et al.*, 1983, 1988).

Colloidal gold method

The preincubation, washes and incubation with specific antisera were similar to the peroxidase methods. The second antibody was anti-rabbit IgG coupled to biotin diluted 1/50 in buffer A. After 1 h at 20 °C, it was washed carefully in buffer B. The sections were finally incubated for 30 min at 20 °C in 1/50 colloidal gold—streptavidin in PBS, and washed extensively in PBS and distilled water.

Controls

The specificities of primary sera were tested previously (see reagents). The possible nonspecific adsorptions of either the antibodies or of the visualizing contrasting agents were tested by replacement of the first antiserum by normal rabbit serum, and/or by replacement of a schedule step by incubation in PBS. The only light nonspecific staining (background) observed was due to adsorption of colloidal gold and, in some cases, of diaminobenzidine. In all experiments, the noise/background ratio was high enough to be interpreted in nuclei (see figures), while it

was difficult to guarantee specificity for cytoplasmic staining.

RESULTS

One cell fertilized oocyte

Most of the eggs obtained 17 h after a second insemination, *i.e.* 15 h after the expected ovulation, displayed 2 centrally placed pronuclei in contact (Figs. 1, 2, 4 and 5) containing large and well delimited pseudo-nucleoli in most sections (Figs. 2, 3, and 5).

Following immunocytochemistry for histones H2B, H3 and H4, all the heterochromatin-containing nuclear structures were specifically labelled (Figs. 2, 3, 4, 5). These structures were located mainly at the border with the nuclear envelopes in the zone where the two pronuclei were in contact (Figs. 2, 4, 5). Usually, at that stage, the chromatin was more labelled, for all tested histones, in one of the pronuclei (Figs. 2, 4). However, owing to the necessarily poor preservation of cytoplasm for immunocytochemistry, it was impossible to find any flagellum indicating the male pronucleus. The chromatin of polar bodies was heavily labelled with anti-H3 antisera.

The pseudo-nucleoli were uniquely but strongly labelled for H2B (Figs. 2, 3). Other histones were found to be absent when tested with the specific antisera utilized (Fig. 5). The "intranuclear chromatoid clusters" of different sizes present in all sections were not stained by the tested anti-histone antibodies (Figs. 2, 5).

As compared to the nuclei, the cytoplasm was poorly labelled for histones,

both with the peroxidase and with the colloidal gold method. However, since no specific labelled organelle and no preferential cytoplasmic localization could be identified, it seems possible to consider this light labelling as background staining.

Four-cell embryos

Four blastomeres were present in all embryos 46 h after the expected ovulation (48 h after the second insemination) (Fig. 6). Their nuclei contained peripherally-located heterochromatin that was well labelled by antibodies to the three histones H2B, H3 and H4 (Figs. 7, 8, 9, 10). In addition, the chromatin surrounding the pseudo-nucleoli was also strongly labelled (Figs. 7, 8, 9, 10). The pseudo-nucleoli were still specifically positive for H2B, but the intensity of the reaction was weaker than that present in former stages (Fig. 7; compare with Figs. 2, 3). The "intranuclear chromatoid clusters" were still not stained (Fig. 9), and the light labelling of cytoplasm was also considered as background.

DISCUSSION

The presence of two pronuclei in contact 15 h after the supposed ovulation in the pig is in agreement with the observations of Norberg (1973) and Tománek *et al.* (1986) in the same species, and with those of Tesarik and Kopečný (1988) in humans or Crozet (1988) in the sheep.

Despite the fact that the nuclei of spermatozoa are devoid of nucleoli, these structures were already present in both pronuclei in the early stages, *i.e.* before fusion. However, the structures of nucleoli in young embryos were unusual, and were similar to "nucleoli" described in oocytes from large follicles (Antoine *et al.*, 1988). They were larger than "ordinary" nucleoli and well delimited. They displayed homogeneous contents without any granules or individual fibrils. The "nucleoli" were composed of densely packed fibrils (Crozet *et al.*, 1986). In oocytes of antral follicles, the "nucleoli" might be composed of acid proteins (Antoine *et al.*, 1988) or, on the contrary, be rich in basic proteins (Takeuchi, 1986). The present demonstration of histone H2B, in the absence of H3 and H4 in these structures, is quite surprising since

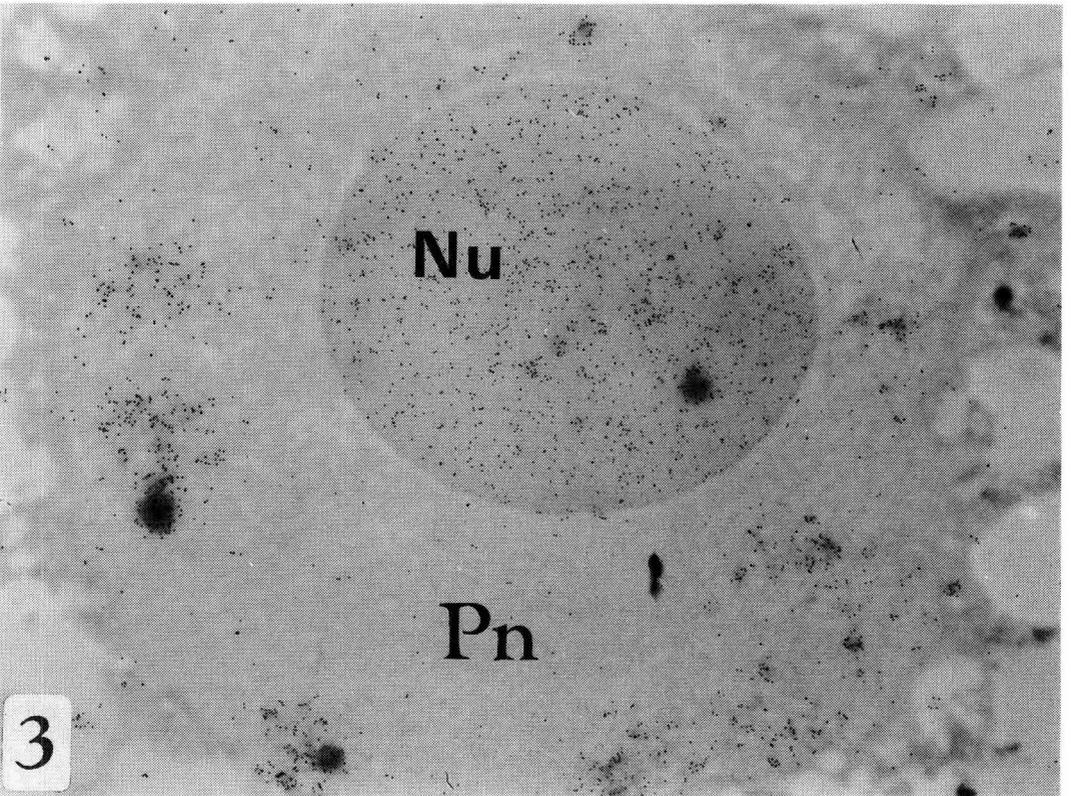
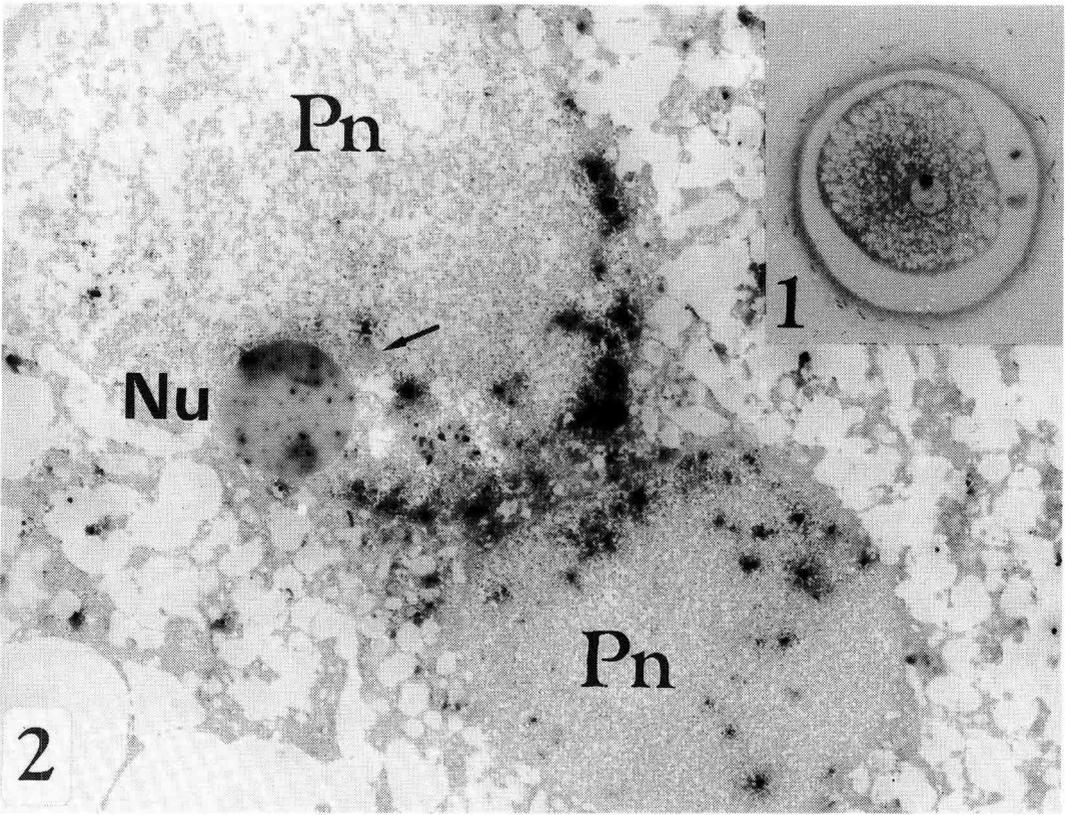
Figs. 1—5. Demonstration of histones H2B, H3, and H4 in pronuclear stages of porcine eggs 15 h after ovulation.

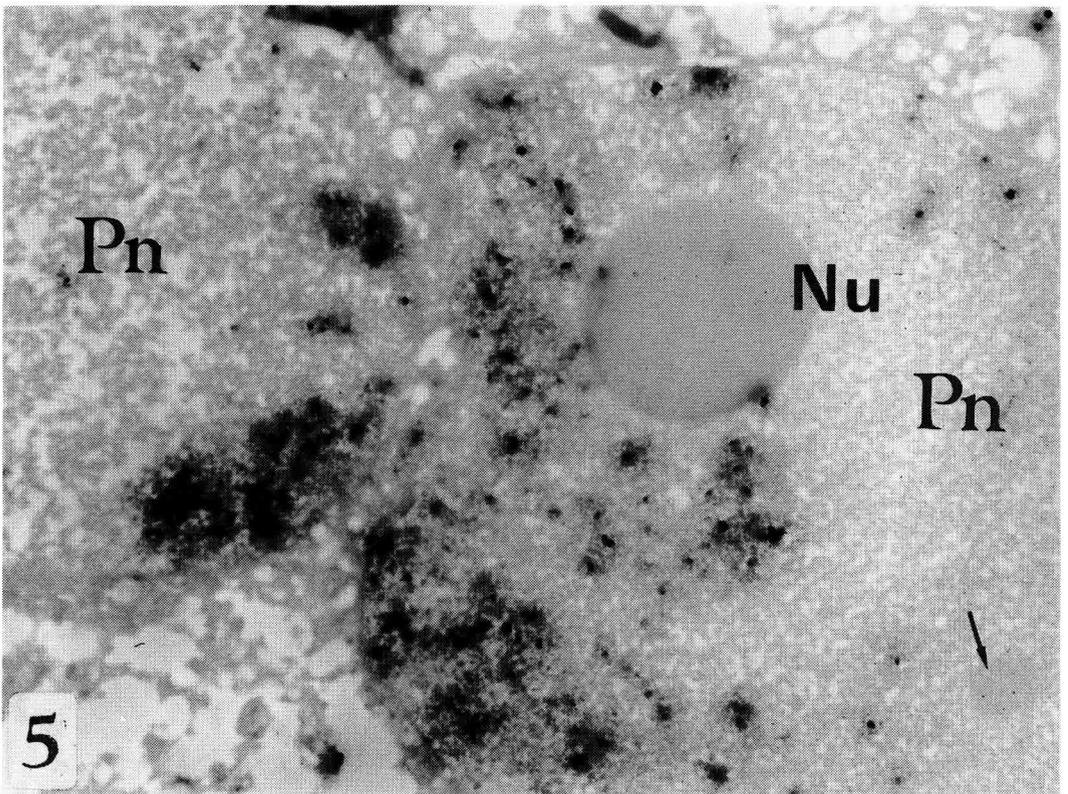
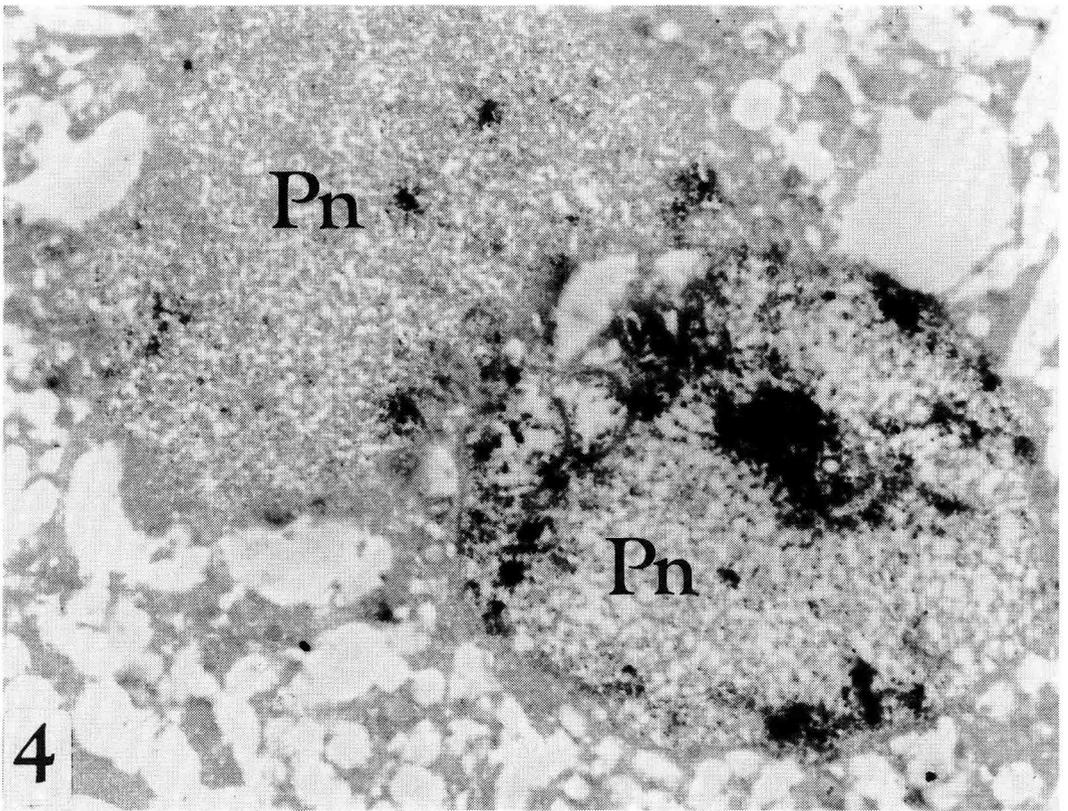
Fig. 1. Semithin section stained with Toluidine blue. (x 500).

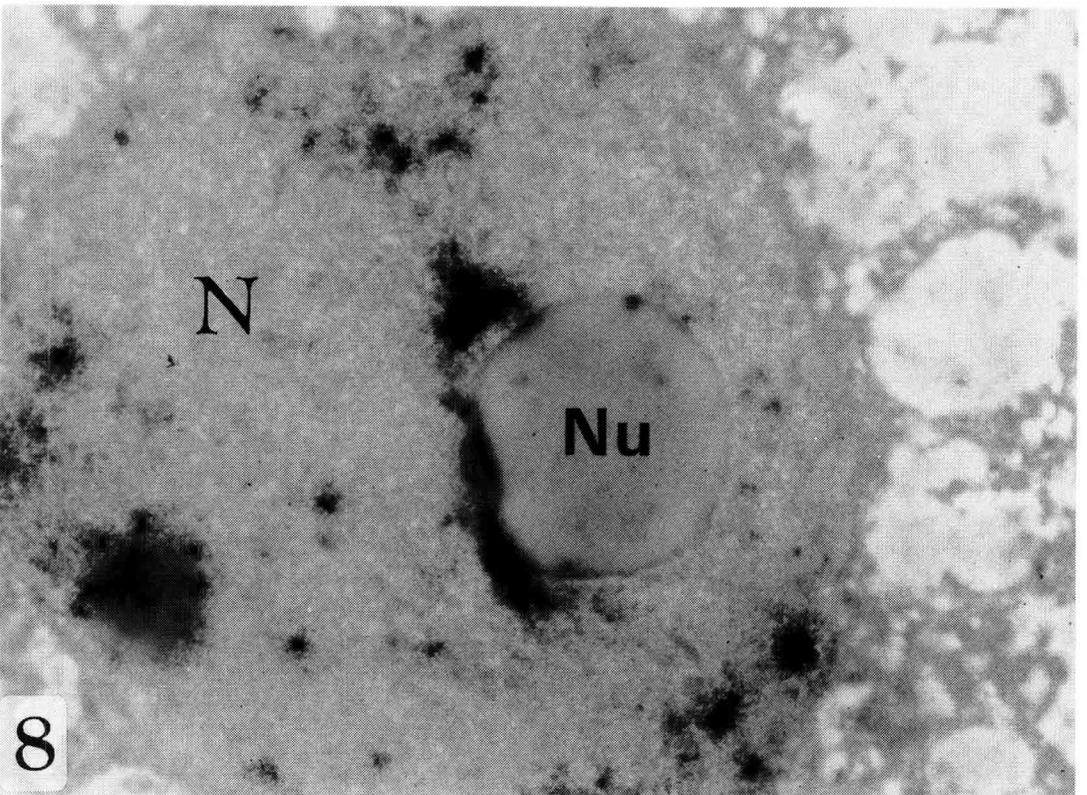
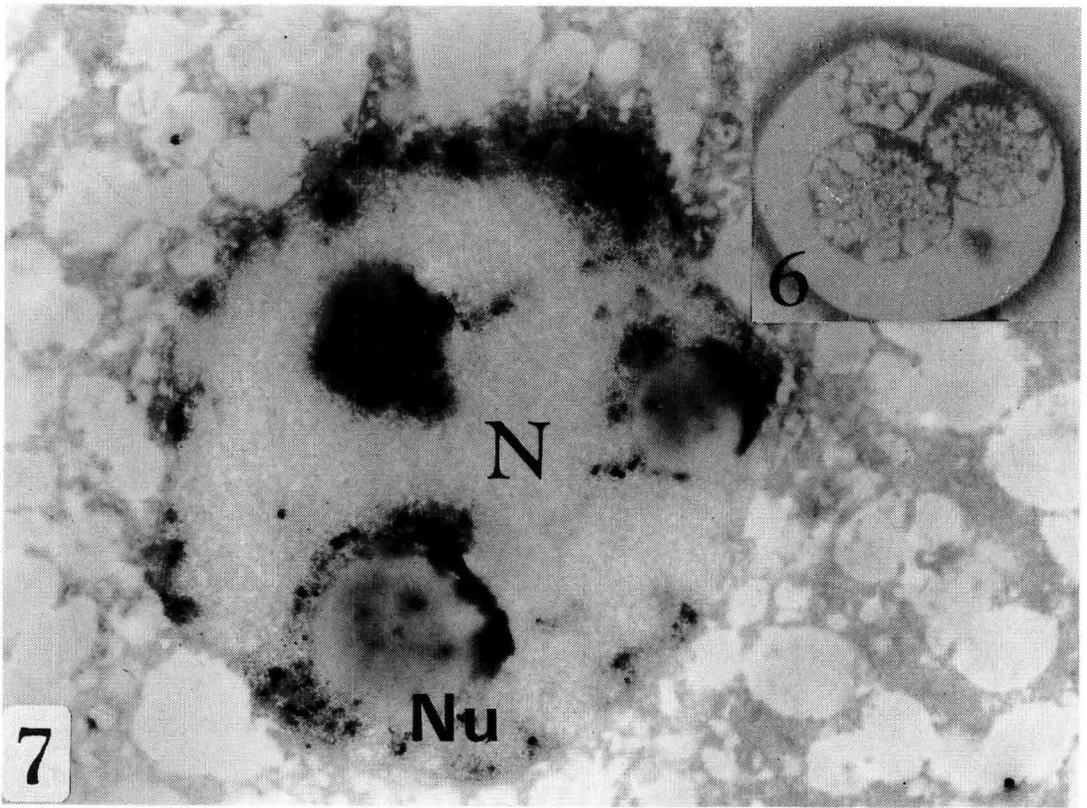
Figs. 2, 3. Demonstration of H2B with peroxidase (x 4 400) and colloidal gold (Fig. 3, x 11 000). The two pronuclei (Pn) are in the center of the egg. Most heterochromatic structures are labelled, and the labelling is intense in the contact zone of both pronuclei. The pseudo-nucleoli (Nu) also contain H2B revealed with both techniques (compare with Fig. 5). Some parts of heterochromatin, such as the intranuclear chromatoid clusters (*arrow*), are not labelled.

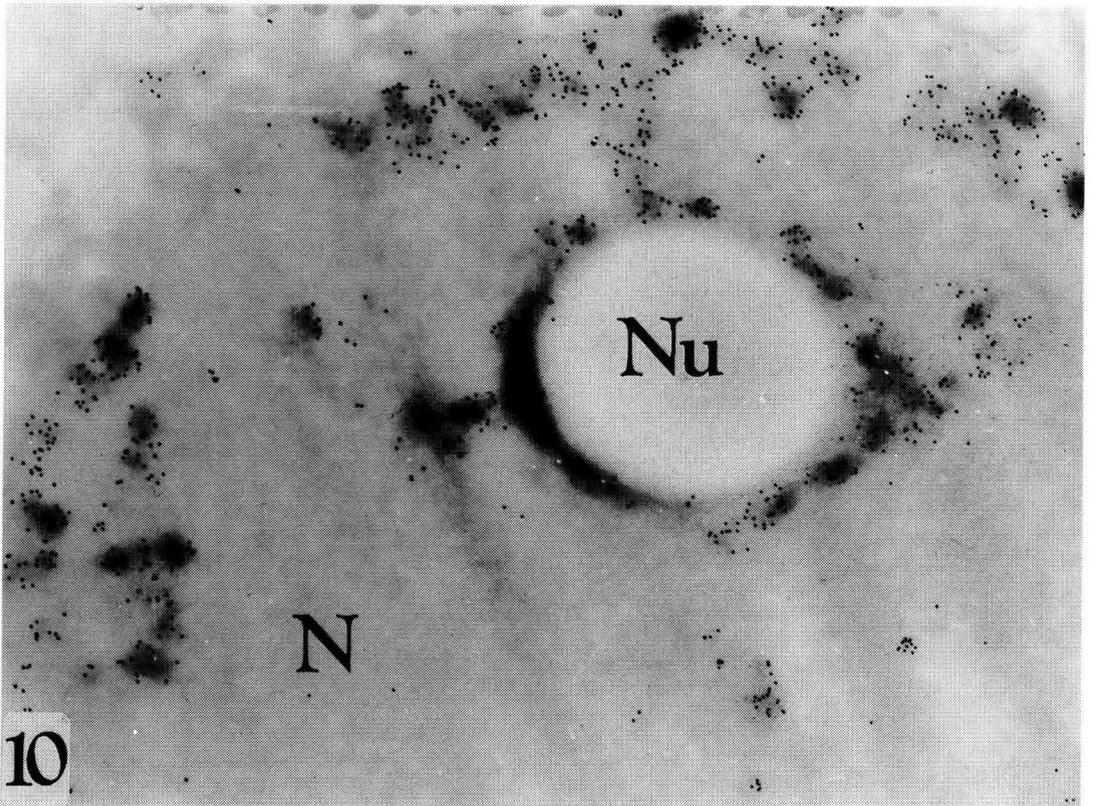
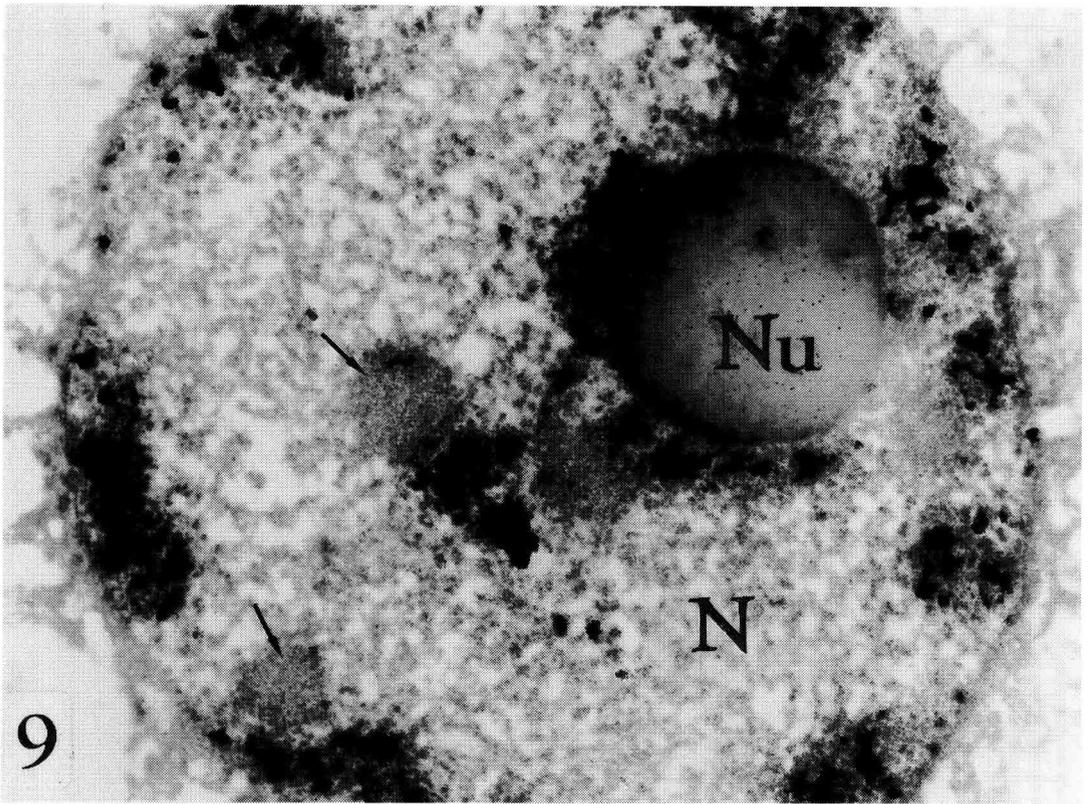
Fig. 4. Demonstration of H3 : the labelling is over the heterochromatin and around the pseudo-nucleoli. The upper pronucleus is only positive in the contact zone. (x 7 600).

Fig. 5. Demonstration of H4 : the labelling is almost similar to that for H2B, with the exception of the pseudo-nucleoli (Nu) that are negative (compare with Figs. 2, 3). The intranuclear chromatoid clusters (*arrow*) are not labelled (x 7 800).









these nucleoproteins are generally associated in core particles. The decline in labelling intensities for H2B in 4-cell pseudo-nucleoli leads to the hypothesis that pseudo-nucleoli could be implied in storage, in the absence of demonstrated synthesis, as they are for ribonucleoproteins (Geuskens & Alexandre, 1984).

The stored histone could be used later, supplying the active chromatin close to the nucleoli (Tománek *et al.*, 1986, 1988) in raw material for new nucleosomes.

The very low labelling of cytoplasm obtained in the present work does not mean that cytoplasmic synthesis did not occur. As observed in spermatids, the turnover of proteins could be rapid and associated with a transport from the cytoplasm towards the nuclei too rapid to be detected by immunocytochemistry (Courtens *et al.*, 1983). Kopečný and Pavlok (1984) demonstrated that synthesis of arginine-rich proteins was present at the time of the decondensation of the spermatozoa. A further demonstration that these proteins correspond to histones is needed.

The fact that one of the pronuclei was always more intensely labelled for all tested histones is intriguing. If one considers the study of Da-Yuan & Longo (1983) that the nucleoproteins are better demonstrated in the male than in the female pronucleus after staining with ammoniacal silver,

then it could be hypothesized that newly-synthesized proteins, possibly histones, preferentially gain the male pronucleus.

On the other hand, the appearance of bowl-shaped regions in the contact zone of pronuclei are suggestive of an exchange of histones between the two nuclei. In the latter hypothesis, it would be more economic for the young embryo to use, at least in part, the nuclear prestored maternal histones.

Since the total male DNA is rapidly devoid of protamine(s) after fertilization (Kopečný & Pavlok, 1975), it is tempting to speculate that the DNA was bare for some time. The reorganization of a typical nucleus with both euchromatin and heterochromatin supposes that different nucleoproteins are associated with specific regions of the genome. Histones which are preferentially associated with heterochromatin and sites active in transcription (Tománek *et al.*, 1986), provided they are close to H1 units (Banchev *et al.*, 1988), are only one of these nucleoproteins. We have demonstrated in this study that as soon as they appeared in nuclei, they were associated with, or determined the appearance of heterochromatin, probably in core particle units, while the rest of the chromatin was mostly unlabelled. One of the many open questions concerns the protein status of these unlabelled areas of the chromatin and their roles in the early embryo.

Figs. 6—10. Demonstration of histones H2B, H3, and H4 in 4-blastomere porcine embryos.

Fig. 6. Semithin section stained with Toluidine blue. 48 h after ovulation. (x 450).

Fig. 7. Demonstration of H2B : some labelling is still present in pseudo-nucleoli (**Nu**). The labelling in the heterochromatin is strong at the periphery of pseudo-nucleoli and at the periphery of the nucleus (**N**). (x 10 000).

Fig. 8. Demonstration of H3 : some heterochromatin, and the periphery of pseudo-nucleoli (**Nu**) are labelled. (x 14 000).

Fig. 9. Demonstration of H4 : the periphery of nuclei (**N**) and pseudo-nucleoli (**Nu**) are strongly positive. The intranuclear chromatoid clusters (arrows) are unlabelled (x 11 000).

Fig. 10. Demonstration of H4 with colloidal gold. The labelling is equivalent to that in Figure 9. Note that the pseudo-nucleolus (**Nu**) is totally negative (x 11 000).

As compared to nuclei of adjacent corona cells which were strongly labelled in the same sections, the staining intensities of nuclei was poor in the embryo, even in the 4-cell stages. The distribution of the histones was also different. Most of the labelled heterochromatin surrounded the pseudo-nucleoli in parts of the nuclei that were active in transcription (Tománek *et al.*, 1986) and some more condensed parts of the chromatin, *i.e.* the intranuclear chromatoid clusters, were never labelled. The inclusion of further cleavage stages in analysis is required to better understand the nuclear events that take place in early embryos.

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