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Original article

Immunocytochemical localization of cyclin/proliferating cell nuclear antigen (PCNA) in fertilized eggs of mice

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Summary — Immunolocalization of cyclin/PCNA (proliferating cell nuclear antigen) was performed with monoclonal antibody using immunogold methods on ultrathin cryosections of fertilized mouse eggs. Immunolabeling in pronuclei was checked 20, 22, 24 and 26 h after HCG injection. A relation between onset of pronuclei migration (early S-phase) and appearance of colloidal particle clusters was found. Afterwards, (mid S-phase) the increase of labelling and the localization of cyclin/PCNA were found throughout the pronuclei, except in the nucleolar bodies. Lower labelling appeared at the time of close reciprocal pronuclei contact (late S-phase). It is concluded that bulk and distribution of cyclin/PCNA in pronuclei is closely related to the progression of first interphase after fertilization.

cyclin / PCNA / DNA synthesis / immunocytochemistry / fertilized mouse egg

Résumé — Localisation immunocytochimique de cycline/antigène nucléaire de cellule en prolifération (PCNA) dans les œufs fécondés de souris. *L'immunolocalisation de cycline, antigène nucléaire de prolifération cellulaire (PCNA) a été effectuée avec un anticorps monoclonal à l'aide d'une technique à l'or colloïdal sur des coupes fines à congélation d'œufs fécondés de souris. Le marquage des pronoyaux a été observé à 20, 22, 24 et 26 h après l'injection d'hCG. Un rapport a été trouvé entre le début de la migration des pronoyaux (début de la phase S) et l'apparition d'amas de particules colloïdales. Ensuite, une augmentation du marquage et la localisation de la cycline (PCNA) ont été observées dans les pronoyaux tout entiers, à l'exception des corps nucléolaires (milieu de la phase S). Le marquage devient plus faible lors du rapprochement des pronoyaux (fin de la phase S). Nous concluons que la masse et la distribution de cycline (PCNA) dans les pronoyaux sont étroitement reliées à la progression de la première interphase après la fécondation.*

cycline (PCNA) / synthèse d'ADN / immunocytochimie / œuf fécondé de souris

INTRODUCTION

In the zygote, DNA replication is one of the most important manifestations of chromatin activation after fertilization. DNA replication in cells is catalyzed by a multiprotein complex containing different enzymes and accessory proteins. One of them, PCNA (proliferating cell nuclear antigen), also called cyclin, was identified as a nuclear acidic protein (36 kDa) in proliferating cells (Bravo *et al,* 1981). Localization but not synthesis of cyclin/PCNA was found to be dependent on DNA replication (Bravo and Macdonald-Bravo, 1985). Cyclin/PCNA is an auxiliary protein for DNA polymerase delta and the protein is localized at nuclear sites of DNA synthesis, thus establishing a close correlation between the nuclear cyclin/PCNA content and DNA replication (Celis and Celis, 1985; Bravo, 1986; Celis *et al,* 1986; Bravo *et al,* 1987; Downey *et al,* 1990).

Incorporation of ^{3}H-thymidine, an indicator of DNA synthesis, was detected in fully developed pronuclei of preimplantation embryos (Oprescu and Thibault, 1965; Tesarik and Kopecny, 1989).

The aim of the present work was to study the immunocytochemical localization of cyclin/PCNA in mouse oocytes fertilized *in vivo*.

MATERIAL AND METHODS

Female F1 (C57/BL6 x CBA) hybrid mice were superovulated with ip injection of PMSG (10 IU) followed 46–48 h later with hCG (10 IU) and mated with males of the same strain. Fertilized eggs were collected from the oviduct at 2-h intervals between 20–26 h post-hCG injection. Immediately after recovery they were observed using Nomarski interference microscopy for presence of pronuclei and those without pronuclei were excluded. Eggs were fixed in 2% paraformaldehyde and 0.2–0.3% glutaraldehyde mixture in phosphate buffer for 90–120 min at 4 °C. Samples were frozen in liquid nitrogen after cryoprotection for 20 min at 4 °C. Samples were frozen in liquid nitrogen after cryoprotection for 20 min with 2.1 M sucrose. Immunolabelling was performed on ultrathin cryosections deposited on EM grids covered with Formvar-carbon film. For labelling the grids were incubated by floating sections for 30 min on diluted primary antibody and 25 min with gold-labelled secondary antibody at room temperature. All sections were preincubated with 1% BSA for 15–30 min. The sections were stained with uranyl acetate and finally embedded in methylcellulose (Griffiths *et al,* 1984; Tokuyasu, 1980, 1986; Raska, 1988). Mouse monoclonal antibody to cyclin/PCNA (Boeringer Co, Mannheim) was used as primary antibody. This antibody reacts specifically with cyclin/PCNA (36 kD. protein) from man, rabbit and mouse. Colloidal gold (10 nm) complexes with goat anti-mouse Ig (Janssen Life Science, Beerse, Belgium) were used in indirect immunolabel method. Tests of label specificity were carried out omitting the primary antibody or secondary antibody. The incidence of gold particles over pronuclei and cytoplasm was evaluated semi-quantitatively (+ sporadic; ++ few; +++ many gold particles or clusters).

RESULTS

At 20 h post-hCG, pronuclei were visible in fertilized eggs (fig 1A). Immunolabelling of cyclin/PCNA was found mainly in the perinuclear area, as indicated by individual gold particles. A few particles were visible over whole pronuclei (fig 2).

At 22 h post-hCG, pronuclei were still localized in the periphery of the cytoplasm (fig 1B). Clusters of gold particles became confined to the perichromatin area for the first time (fig 3).

At 24 h post-hCG, pronuclei were found in the centre of fertilized eggs, but they were not yet in close contact (fig 1C). The incidence of gold particle clusters was found to be higher than at 22 h post-hCG

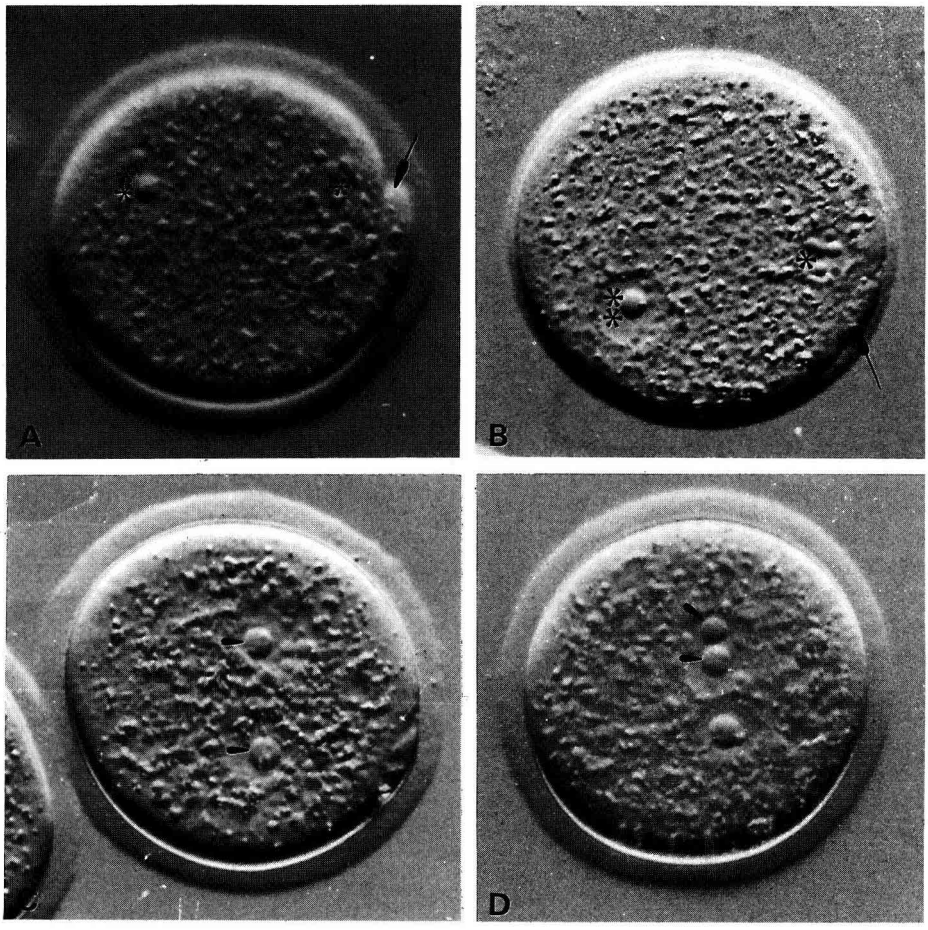
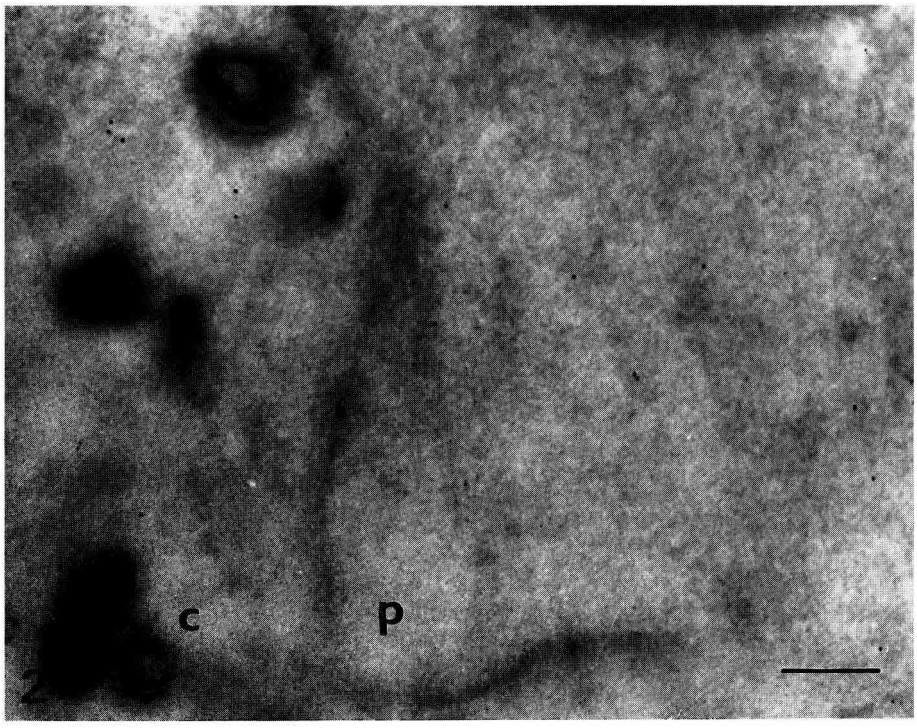


Fig 1. Light microscopy of fertilized mouse egg. A. 20 h post-hCG injection; B. 22 h post-hCG; C. 24 h post-hCG; D. 26 h post-hCG. Male pronucleus: doubles star; females pronucleus: star; nucleolar precursor: arrowheads; second polar body: arrow.

(fig 4). At the same time labelling was also observed over the perinuclear chromatin area. Only sporadic colloidal particles (not significant) were localized over the nucleolar body (fig 5).

At 26 h post-hCG, pronuclei were in close contact and had prominent nucleoli (fig 1D). A total decrease of immuno-gold particles over whole pronuclei was evident, but a higher concentration remained on the cytoplasm (*vs* nucleus) (fig 6). Figure 7 shows absence of labelling in control experiment. The semi-quantitative results are summarized in table I.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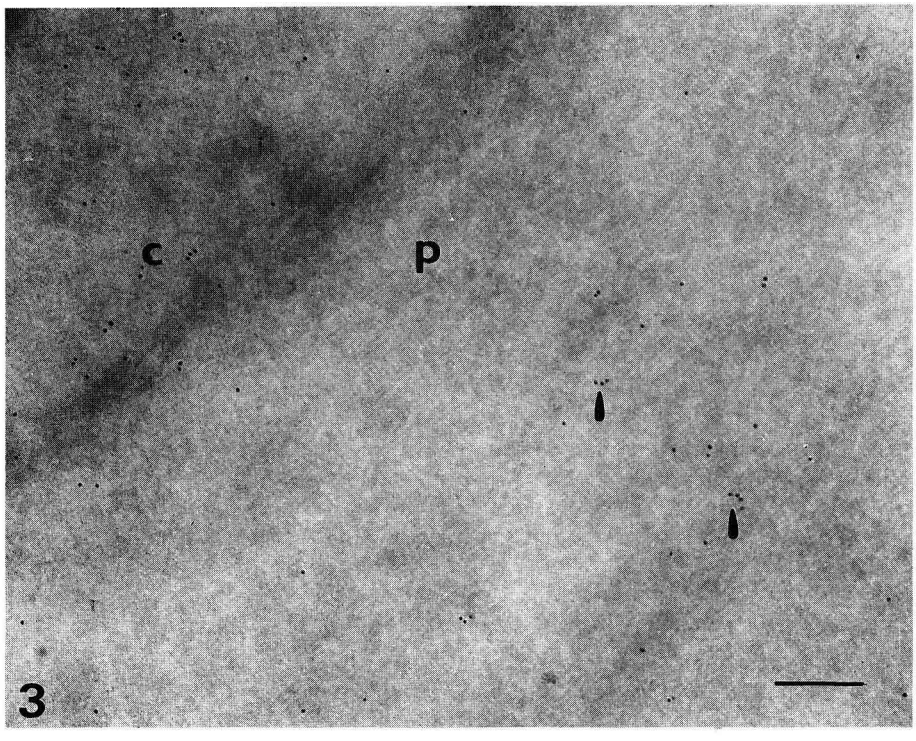
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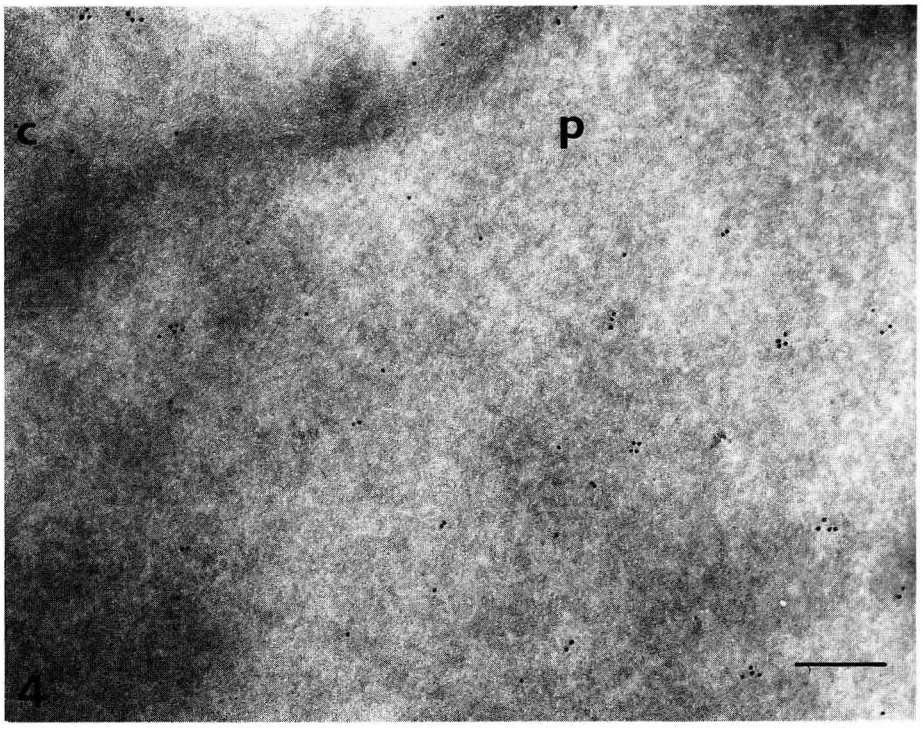
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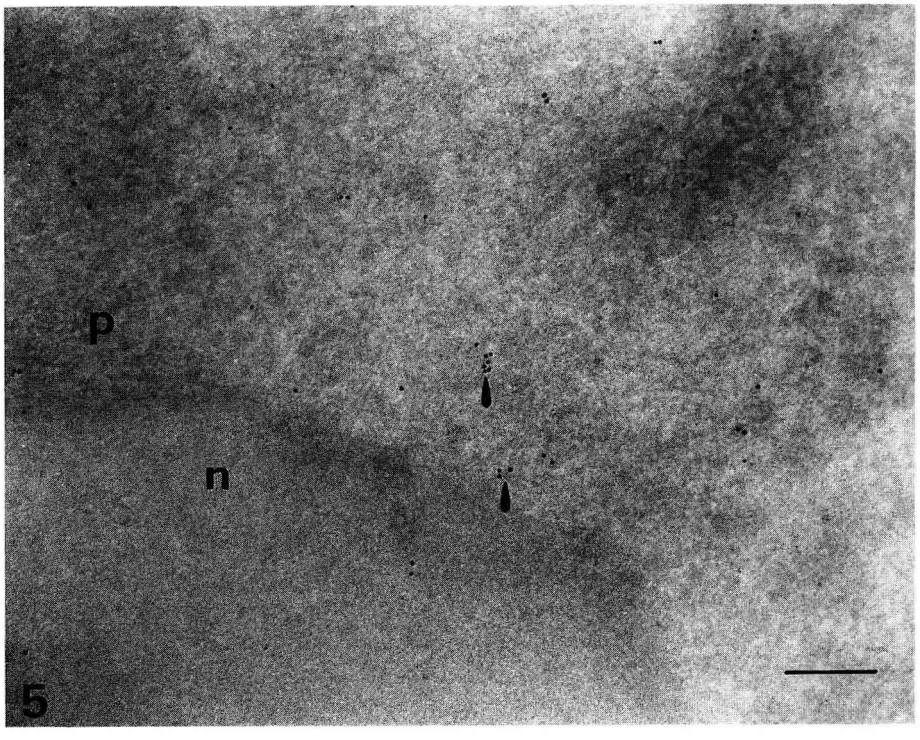
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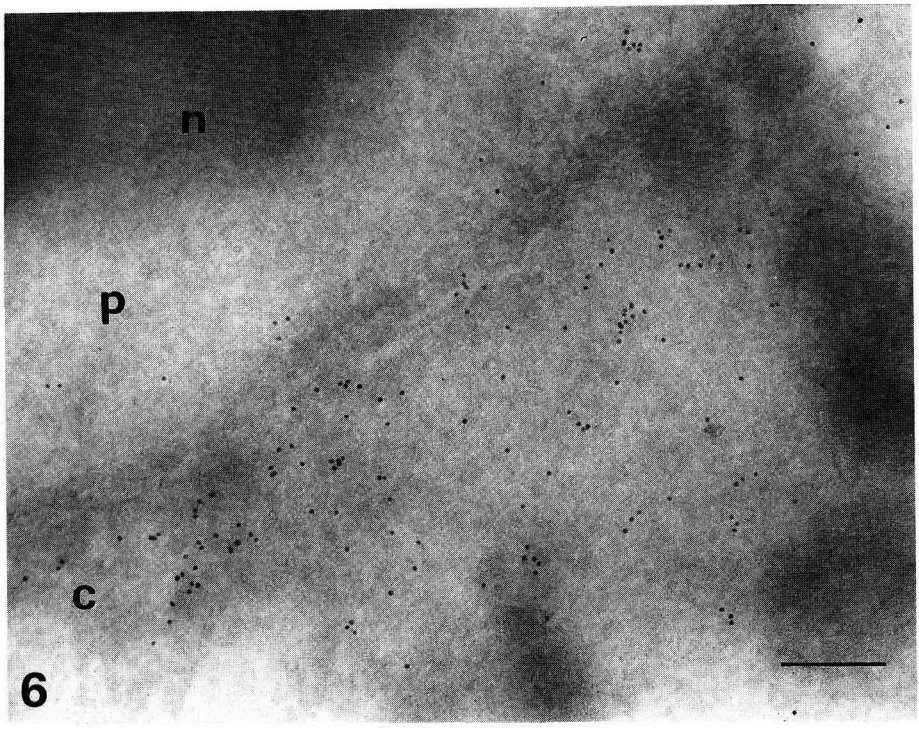
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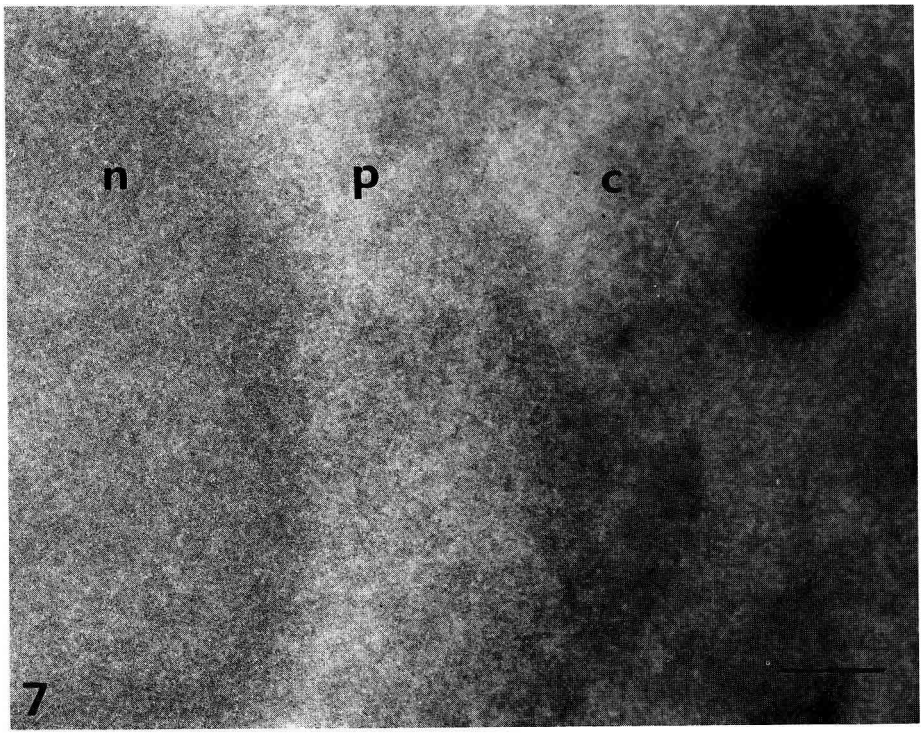
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Table I. Semi-quantitative results of cyclin/PCNA labelling.

Hours after hCG	*Pronucleus*	*Cytoplasm*
20	+	++
22	++	+++
24	+++	+++
26	++	+++

Labelling: +: sporadic; ++: low; +++ high (clusters).

DISCUSSION

In this study we have attempted for the first time to localize cyclin/PCNA in relation to the course of S-phase in fertilized mouse eggs. Formation and localization of pronuclei in fertilized eggs served as a criterion for selection of individual stages of S-phase. DNA synthesis begins after formation of pronuclei, continues during the migration of the centre of cytoplasm and finishes when pronuclei are in close contact (Sathanathan, 1984; Bürki, 1986). As was noted earlier, (see *Introduction*), DNA replication is closely related to cyclin/PCNA distribution. Our results are in line with data showing a nuclear concentration of cyclin/PCNA during the S-phase of cell cycle in other cell types (Bravo and Macdonald-Bravo, 1985; Bravo, 1986; Celis *et al,* 1986). It seems that there is a relationship between the amount of nuclear cyclin/PCNA and DNA synthesis (Bravo and Macdonald-Bravo, 1987). From their studies on immunolocalization of cyclin/PCNA in synchronized cultured cells, Raska *et al* (1989) proposed that the clusters of gold particles, situated mainly in the perichromatin regions, identify sites related to DNA synthesis (transcription sites). We cannot

Fig 2. Fertilized mouse egg labelled by cyclin/PCNA antibody 20 h post-hCG injection. Individual gold particles are found mainly in the perinuclear region of the pronucleus: p. A few particles can be visible over whole pronucleus. c: cytoplasm; bar = 200 nm.

Fig 3. Immunolabelling of cyclin/PCNA in fertilized mouse egg 22 h post-hCG injection. First occurrence of gold clusters (arrowheads) in the chromatin regions was observed. These clusters were localized mainly in the perichromatin area. c: cytoplasm; p: pronucleus; bar = 200 nm.

Fig 4. Part of the pronucleus of fertilized mouse egg 24 h post hCG injection. Cyclin/PCNA labelling expressed by increase occurrence of gold clusters throughout the pronuclus: p; c: cytoplasm; bar = 200 nm.

Fig 5. Another pronucleus of fertilized mouse egg 24 h post-HCG injection. Gold clusters (arrowheads) were also observed in the perinucleolar area. Few individual gold particles are visible in the nucleolar precursor: n; p: pronuclear area; bar = 200 nm.

Fig 6. Picture of immunolocalization of cyclin/PCNA in fertilized mouse egg 26 h post-hCG injection. A decreased immuno-signal was observed in the pronucleus: p. A high number of gold particles was retained in the cytoplasm: c; n: nucleolar precursor.

Fig 7. Labelling control by omitting of the primary antibody. The same picture can be observed after incubation with gold particles free of secondary antibody. p: pronucleus; n: nucleolar precursor; c: cytoplasm; bar = 200 nm.

confirm this specific localization because cryosections provide a lower quality picture of cell ultrastructure than sections of embedded material (Lowicryl or Epon). A high amount of gold particles persists in the cytoplasm at all stages studied. It should be emphasized that our fixation procedure (paraformaldehyde–glutaraldehyde) could also reveal free cyclin/PCNA unbound to replication complexes (Bravo *et al*, 1987).

Our results demonstrate for the first time changes in cyclin/PCNA content and distribution in mouse pronuclei during first interphase. In addition, cyclin/PCNA molecules appear located in/or close to dense chromatin. We suggest that the increased amount and redistribution of labelled cyclin/PCNA during S-phase is related to DNA synthetic activity.

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