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Original Article

Changes in embryonic 8-cell nuclei transferred by means of cell fusion to mouse eggs

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ABSTRACT Metaphase II and activated mouse oocytes were fused with 8-cell blastomeres, and morphological changes in the transferred nuclei were followed using light and electron microscopy. In metaphase II oocytes, blastomere nuclei underwent premature chromosome condensation (PCC) typical for S-phase nuclei: chromatin pulverization. Then an abortive spindle was formed without evident microtubule organizing centers. Blastomere chromosomes condensed to a lesser degree than meiotic chromosomes and lacked mature functional, trilaminar kinetochores. After parthenogenetic activation of these oocytes, blastomere chromosomes followed, in synchrony with oocyte chromatin, a similar route of changes (anaphase, telophase) and then reformed interphase nuclei of the pronuclear type. Remodeling of 8-cell nucleus thus occurred, but the integrity of the chromatin set was frequently disturbed by formation of micronuclei. If blastomere fusion with oocytes was done close to activation (either before or after parthenogenetic stimulation), the chances of remodeling of the nuclei decreased, because PCC was not regularly induced in all oocytes. In hybrids produced 60 min or later after oocyte activation, blastomere nuclei were maintained in interphase without any structural modifications. Multiple experiments in the mouse have shown that the nuclei from 8-cell stage transferred to enucleated oocytes and egg cells are not capable of substituting for pronuclear functions. Possible reasons for impaired functional reprogramming of 8-cell nucleus in the mouse are discussed in light of our present findings on the morphology of nuclei transferred before and after oocyte activation.

KEY WORDS: *mouse oocyte, nuclear transfer, nuclear remodeling, PCC*

Introduction

Nuclear transfer in mammals has become a challenge for experimental embryologists for at least two reasons: 1) as a tool for producing genetically identical animals; 2) as a system for analysing development. In the first approach, experimental success is measured by a number of manipulated embryos which develop to term. In the second, attention is focused on the functioning of transplanted nucleus in the host cytoplasm. Analysis of various nucleo-cytoplasmic combinations with restricted developmental capabilities may be as important as studies on effective combinations ensuring normal development.

Experiments in the mouse have repeatedly shown that the capability of transcriptionally active embryonic nuclei of supporting development of reconstituted embryos depends on the stage of the recipient cytoplasm. Enucleated mouse zygotes receiving the nuclei from early cleavage stages arrest during preimplantation development (McGrath and Solter, 1984), whereas enucleated 2-cell blastomeres receiving similar nuclei may continue development up to term (Tsunoda *et al.*, 1987).

Why embryonic nuclei fail to cooperate with egg cytoplasm in the mouse is intriguing. One may assume that developmentally older nucleus cannot substitute for pronuclear functions because cytoplasmic factors responsible for reprogramming are lacking in host zygotes. These factors are supplied by meiotic oocytes, since the nuclei from late 2-cells, transplanted to meiotic cytoplasts, are capable of supporting development up to term (Kono *et al.*, 1991).

The process of nuclear reprogramming cannot be understood without analysing nuclear events going on in transplant embryos reconstructed from various donors and recipients. In the present work we monitored changes of 8-cell embryonic nuclei transferred by blastomere fusion to metaphase II oocytes and parthenogenetic

Abbreviations used in this paper: 1/8 nuclei: 8-cell stage nuclei; PCC, premature chromosome condensation; PC-nucleus, prematurely condensed nucleus; MPF, maturation promoting factor; NE, nuclear envelope; MTs, microtubules; MTOCs, microtubule organizing centers; LM, light microscopy; EM, electron microscopy.

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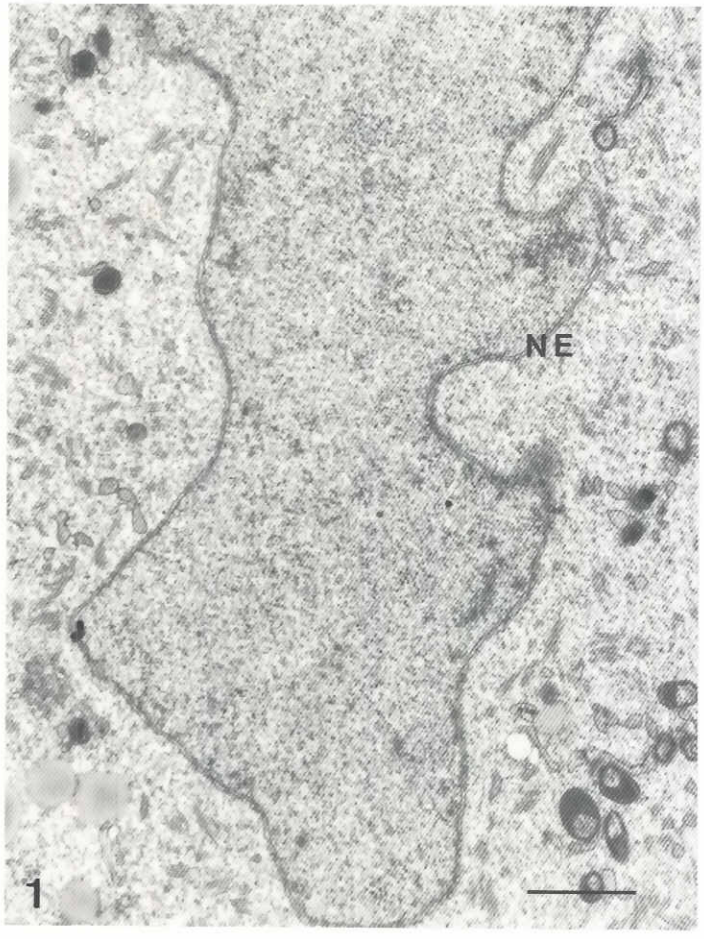
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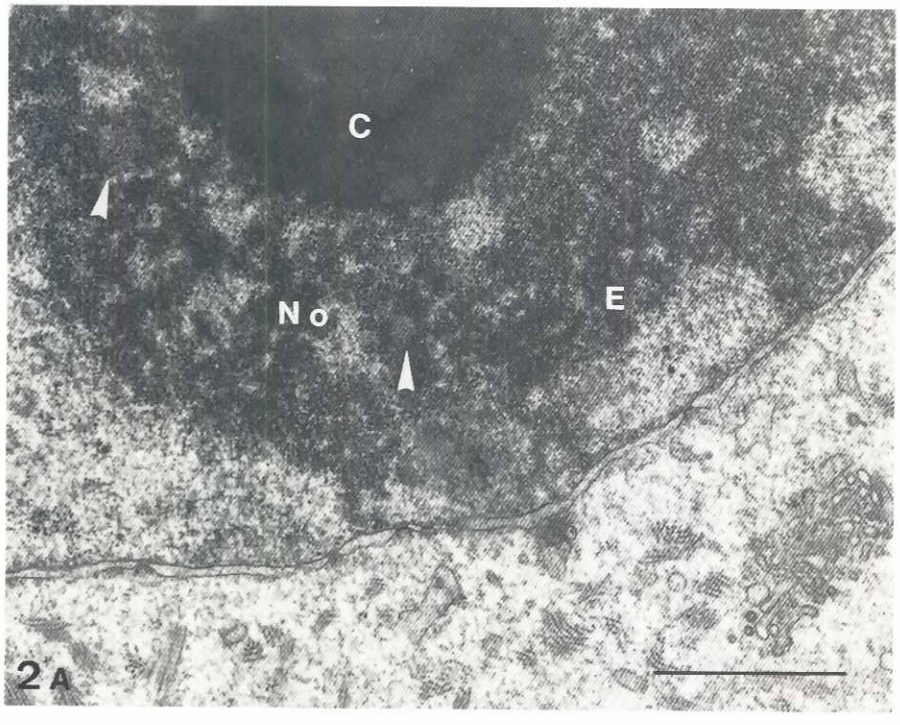
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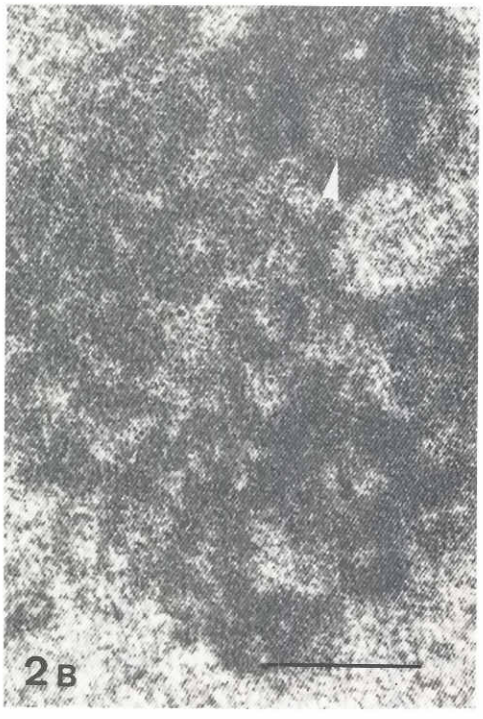
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egg cells. We produced hybrid cells rather than typical nuclear transplants using enucleated cytoplasts, because in the first combination the morphology of the blastomere nucleus can be directly related to the control oocyte/egg nucleus retained by the hybrid cell. Moreover, our previous studies on oocyte-thymocyte hybrids (Czolowska *et al.*, 1984; Szöllösi *et al.*, 1986a,b, 1988) together with studies on heterokaryons formed between blastomeres and egg cells (Dyban *et al.*, 1988; Kubiak *et al.*, 1991) provide good comparative data for interpretation of nuclear events occurring before and after activation of mouse oocyte. Possible side effects brought about by the introduction of the blastomere cytoplasm can be neglected, because intact zygotes of mouse receiving 8-cell cytoplasm develop normally (Barnes *et al.*, 1987). Toxic effects of polyethylene glycol used in our experiments for cell fusion are not manifested during the first cell cycle, neither at EM level (our present and previous observations) nor in the potential of PEG-treated egg cells to enter the first mitotic cycle (Ciemerych, personal communication).

Results

Morphology of nuclei from 8-cell embryo

Nuclei of 8-cell stage blastomeres (= 1/8 nuclei) are almost devoid of heterochromatin. Nuclear envelope (NE) has irregular outline and occasionally forms deep indentations (Fig. 1). Large nucleoli (3-5 per nucleus) have a bipartite structure and are composed of a compact fibrillar portion and a peripheral, asymmetrically arranged, fibrillo-granular part (Fig. 2A,B). Intranuclear annulate lamellae are frequently met in the nucleoplasm, and sporadically associated with the inner leaflet of NE.

Premature chromosome condensation of blastomere nuclei in metaphase II oocytes

In metaphase II oocytes, blastomere nuclei undergo premature chromosome condensation (PCC) (Fig. 3) under the activity of the maturation promoting factor (MPF) present in meiotic oocytes (Masui, 1985; Sorensen *et al.*, 1985; Hashimoto and Kishimoto, 1988). In LM preparations, PCC was initiated 15 min after fusion in about half (7/12) of cell hybrids, and 30 min after fusion PCC occurred in all 17 hybrids inspected. One hour after fusion most hybrids studied with LM contained a distinct spindle area with PC-chromosomes (10/13 hybrids; Fig. 4A,B); in two hybrids PCC spindle could not be recognized, and in one hybrid PC-chromosomes were randomly distributed over the oocyte metaphase II spindle.

Electron microscopy of hybrids fixed 1 h after fusion revealed the presence of a giant, barrel-shaped spindle formed of newly polymerized microtubules (MTs), with prematurely condensed chromosomes aligned in the equatorial region (Figs. 3, 8A) lacking mature functional kinetochores. The spindle was built of more or less regularly arranged bundles of MTs oriented along a main spindle axis. Microtubule organizing centers (MTOCs), which are regularly associated with metaphase II spindle poles in the mouse, were not identified at the poles of the induced spindle. Chromosomes of PC-blastomere nucleus rarely condensed to such a degree as the metaphase II chromosomes. They were typically pulverized (Fig. 4C,D) and at EM level appeared as clumps of differentially condensed chromatin fragments (Fig. 8A,B). Such morphology shows that at the moment of fusion the blastomere nuclei were engaged in DNA replication.

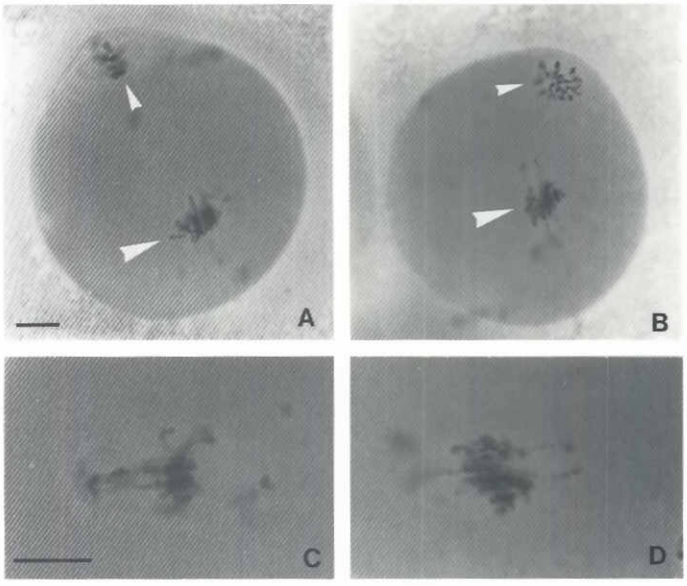


Fig. 4. Premature chromosome condensation (PCC) of blastomere nuclei in metaphase II oocytes. *One hour after fusion 1/8 nucleus transforms into differentially condensed (pulverized) chromosomes distributed over the induced spindle.* **(A,B)** *two heterokaryons showing PCC spindle (long arrowhead) beside the metaphase II spindle (short arrowhead).* **(C,D)** *Two examples of PCC at higher magnification (D is an enlarged view of PCC from 4B). Bars, 10 μm.*

Segregation of prematurely condensed chromosomes after oocyte activation, and reformation of interphase nuclei

In this chapter we describe changes leading to the remodeling of a blastomere nucleus following induced PCC. The time of incubation of 1/8 nuclei in the oocyte cytoplasm was therefore not shorter than 30 min, and oocytes were activated when PCC changes were fairly advanced.

Fig. 1. A fragment of a nucleus of a control 8-cell mouse embryo. *The nucleus is irregular in shape and the nuclear envelope (NE) develops numerous indentations. The chromatin is uniformly dispersed. Bar: 1 μm.*

Fig. 2. Nucleolus of a control 8-cell mouse embryo. (A) *Parallel section of the nucleolus (No) from the blastomere shown in Fig. 1. Nucleolus is associated with NE and has bipartite structure. Internal compact portion (C) is constituted of thin fibrillar elements. An asymmetrically placed fibrillo-granular (E) portion is in peripheral position. Fibrillar centers are marked by arrowheads. Bar: 1 μm.* **(B)** *The fibrillo-granular portion of the nucleolus at higher magnification. Fibrillar center indicated by arrowhead. Bar: 0.5 μm.*

Fig. 3. Premature chromosome condensation (PCC) of the blastomere nucleus in metaphase II oocyte. *Metaphase-like spindle apparatus with unevenly condensed chromosomes. Arrows point to the denser regions of chromosomes. Bar: 10 μm.*

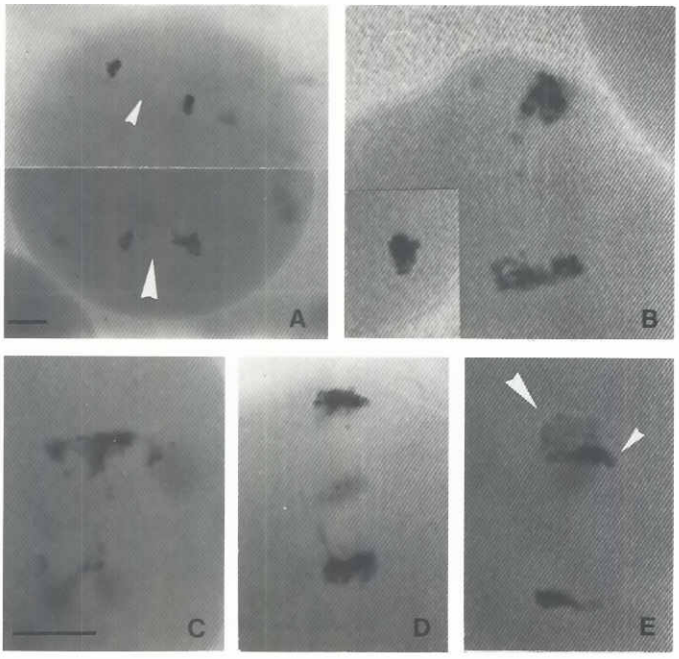


Fig. 5. Karyokinetic division of PCC nuclei following oocyte activation. **(A)** *Two chromatin sets synchronized in telophase. In comparison with telophase II (short arrowhead), 1/8 chromatin (long arrowhead) is far less condensed.* **(B)** *Formation of the false polar body over radially oriented 1/8 telophase spindle (inset shows highly condensed telophase II chromatin from the same heterokaryon).* **(C,D)** *Morphology and segregation of telophase chromatin is influenced by the time of incubation of PCC nucleus in metaphase II oocyte (C= 30 min, D= 45 min) before activation.* **(E)** *After activation 1/8 chromatin (long arrowhead) starts to decondense ahead of the oocyte chromatin, which is still compacted (short arrowhead). Second telophase 1/8 nucleus out of focus. Time of culture post activation: (A-D) 1 h, (E) 2 h. Bars: 10 μm.*

Parthenogenetic activation of metaphase II oocyte containing PC-chromosomes set, applied 30-60 min after fusion, initiated chromosome movement on both the oocyte meiotic spindle and PCC-induced spindle. In all hybrids inspected with LM one hour after activation, both chromatin groups were synchronized in telophase. The majority of hybrids (11/13) had two telophase spindles: one from the oocyte, and the other from the dividing blastomere nucleus (Fig. 5A,B). In one case karyokinesis of 1/8 nucleus was suppressed, and in the second PC-chromatin intermingled with the oocyte chromatin. Pole-to-pole movement of chromatin on the induced spindle suffered apparently from the fact that the nucleus was prematurely condensed in S-phase. More than two telophase groups could form on an abortive spindle with fragments of pulverized chromatin dispersed along the spindle body and beyond it (Fig. 5C,D). As a rule, the telophase blastomere chromatin was less condensed than the telophase II chromatin (Fig. 5B). This difference was even more obvious in hybrids inspected 2 h after activation (11 hybrids, LM, Fig. 5E). Peripheral karyokinesis of the blastomere nucleus could lead to the formation of a protuberance on the oocyte surface enclosing one telophase group (false polar body, Fig. 5B).

Hybrids cultured for a few hours after activation showed the presence of interphase nuclei reformed from the condensed chromatin. Depending on the original location of PCC set in relation to metaphase II, 1 to 4 nuclei could eventually be present in the activated oocyte (Figs. 6, 7). Reconstructed blastomere nucleus (i) was larger than the female pronucleus and could be identified due to numerous nucleoli.

At the ultrastructural level, the reformed 1/8 nuclei were, in many respects, similar to the egg pronucleus. Their chromatin decondensed to the same degree, and the nucleoli had identical fibrillar composition (Fig. 9). Reformed 1/8 nuclei also initiated and continued «bleb» formation (Szöllösi and Szöllösi, 1988) in synchrony with the female pronucleus. They differed, however, from the egg pronucleus as they contained intranuclear annulate lamellae which are rarely observed in female pronuclei. Some reformed 1/8 nuclei must have been aneuploid since they were associated with a number of micronuclei of different sizes (Fig. 10), originating from dispersed chromosomes of PC-nucleus.

Changes in blastomere nuclei transferred to oocytes at the time of activation

Activation of a metaphase II oocyte leads to the decline of MPF activity, which reaches its lowest level at telophase II (Weber *et al.*, 1991). If a blastomere nucleus is transferred very close to the moment of activation, before or after application of the activating stimulus, either PCC is initiated or interphase is maintained. The reaction of a nucleus is clearly dependent on the activity of MPF operating at metaphase-interphase transition. Although nuclei introduced just before activation are exposed to MPF slightly longer than those transferred after activation, no significant differences between the two variants could be found, because individual

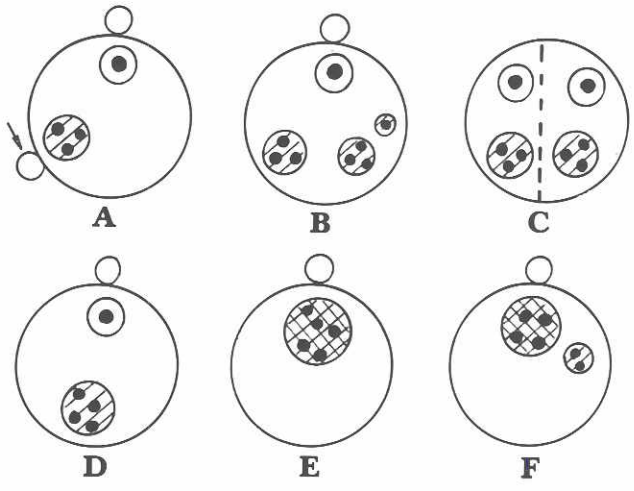


Fig. 6. The development of interphase nuclei in heterokaryons formed between metaphase II oocytes and blastomeres, submitted to activation. *Egg pronucleus: clear circle; blastomere nucleus: striped circle. Nucleoli shown as black dots. Following oocyte activation karyokinetic division of PC-nucleus results in extrusion of a false polar body (arrow) as in* **(A)** *or formation of two blastomere nuclei* **(B,C)** *frequently associated with micronuclei (B). Some heterokaryons (C) divide by immediate cleavage.* **(D)** *Karyokinesis suppressed, one blastomere nucleus.* **(E,F)** *Formation of a hybrid nucleus (squared circle) after complete (E) or partial (F) incorporation of 1/8 chromatin into an egg pronucleus. Second polar body depicted at the top of heterokaryons.*

oocytes react to the activation and complete the second meiotic division with varying dynamics.

In 3 oocytes hybridized with a blastomere 15 min before activation and cultured 1 h after activation, oocyte nuclei were in anaphase or telophase. One oocyte had 1/8 nucleus in PCC: islands of pulverized chromatin were associated with MT bundles of a rudimentary spindle and only immature kinetochores formed (Fig. 11A). In the second oocyte PC-chromosomes were highly condensed, as in a normal metaphase plate and the kinetochore had normal trilaminar structure (Fig. 11B). In the last oocyte 1/8 nucleus had undergone fragmentation (Fig. 12). Its NE was partially intact, and the cytoplasmic organelles penetrated deeply among the chromatin islands. At few μm distance from the fragmented nucleus was a rudimentary telophase spindle made of a few MTs but containing the midbody (Fig. 12).

Diverse changes were also observed in a reverse combination, i.e. when blastomeres were fused with previously activated oocytes. In 4 oocytes fused 30-40 min after activation and inspected 1 h post fusion, the oocyte nucleus was in anaphase/telophase; two hybrids had PC-chromatin in telophase, whereas in the other two 1/8 nuclei were in interphase (Fig. 15A, LM). In one hybrid studied with EM, the oocyte nucleus was in anaphase/telophase transition and a portion of blastomere chromatin migrated to the meiotic spindle; irregularly condensed clumps typical of S-phase chromatin were dispersed along the spindle, and some adjoined the oocyte chromosomes (Fig. 13). PC-chromatin never condensed to a degree typical for meiotic chromosomes.

In the first cycle the period permissive for nuclear envelope breakdown and PCC of embryonic nucleus is very short. We estimate that the critical time separating both treatments, i.e. activation and fusion, within which PCC of a blastomere nucleus could be induced regularly lasts about 15-20 min.

Blastomere nuclei transferred into interphase egg cells

Blastomere nuclei introduced into activated oocytes, after the cytoplasm has lost the chromatin condensation activity remain in interphase. When heterokaryons were formed 1 h or later after egg activation, and cultured for 1-7 h post fusion, the blastomere nuclei were always recognizable due to their irregular shape, intense staining of the nucleoplasm and multiple nucleoli (LM, Fig. 15B-D). At the EM level these nuclei preserved all the features of control blastomere nuclei, which are the folded NE, the presence of intranuclear annulate lamellae, and the character of nucleoli (Fig. 14). The latter were composed of two parts (the compact fibrillar part and the fibrillo-granular part) and had several fibrillar centers (Fakan and Hernandez-Verdun, 1986; Derenzini *et al.*, 1990). The egg pronucleus, in contrast, had a smooth outline and contained one large electron-dense, nucleolus-like body (Fig. 14).

If blastomere nuclei were transferred into eggs between 1 and 2 h after egg activation they underwent swelling (27 hybrids studied with LM, Fig. 15C). Swelling coincided with suppression of pronuclear growth, and after a few hours of culture the blastomere nucleus was some 2-3 times larger in diameter than the egg pronucleus. Swelling occurred notwithstanding whether parthenogenotes extruded the second polar body or not, or divided by immediate cleavage. In contrast, blastomere nuclei transferred later than 4 h after egg activation and incubated for 2-4 h thereafter did not swell (7 hybrids, LM, Fig. 15D).

Both interphase nuclei present in heterokaryons, i.e. the pronucleus and the blastomere nucleus, initiated formation of blebs by NE according to the time-table of one-cell egg (Szöllösi and Szöllösi, 1988).

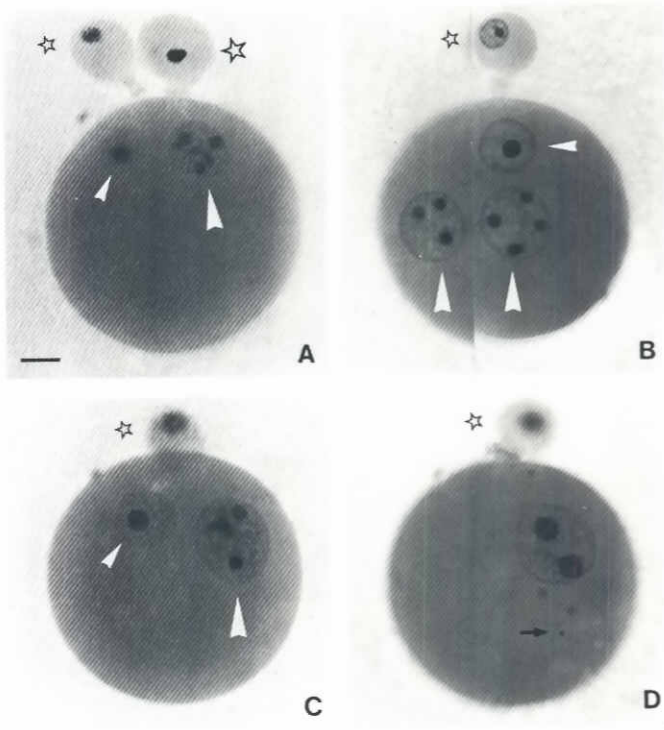
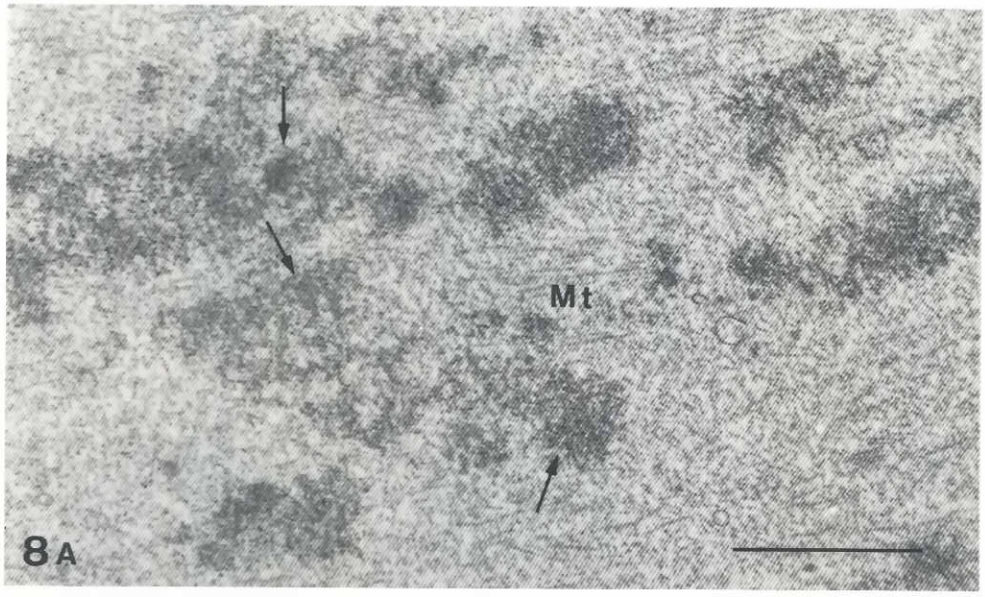


Fig. 7. Formation of interphase nuclei in oocyte-blastomere hybrids submitted to activation (4 h culture after activation). *After induced PCC, pronuclear type interphase nuclei are reconstructed (long arrowhead) beside the egg pronucleus (small arrowhead).* **(A)** *Formation of one nucleus and false polar body (large asterisk), or two nuclei* **(B)**. *In some heterokaryons karyokinesis was suppressed* **(C)**, *whereas in others true hybrid nucleus was formed after incorporation of PCC into metaphase II* **(D)**. *Small asterisk marks the second polar body. Arrow points at a micronucleus. Bar: 10 μm.*

Discussion

Our previous work on nuclear remodeling in the mouse has shown that formation of a pronucleus (or a nucleus which is structurally identical with the pronucleus) from a transferred nucleus requires nuclear envelope breakdown and chromatin condensation — two events that can be readily induced by metaphase II oocytes (Czolowska *et al.*, 1984; Szöllösi *et al.*, 1986a,b, 1988). Exposure of a donor nucleus to an oocyte cytoplasm and the resulting chromatin condensation are important prerequisites for nuclear reprogramming and have beneficial effects on the development of nuclear transplant embryos, as was demonstrated in experiments with amphibians (DiBerardino, 1988) and mammals (rabbit: Collas and Robl, 1991; Collas *et al.*, 1992a,b). During the condensation process in mitosis most of the nuclear proteins, except for histones, are removed from the nucleus (Nagl, 1985; Kellenberger, 1987; Alberts *et al.*, 1989). Cytoplasmic proteins now have free access to chromosomes and may be passively trapped by the telophase nucleus as the nuclear envelope reforms (Swanson and McNeil,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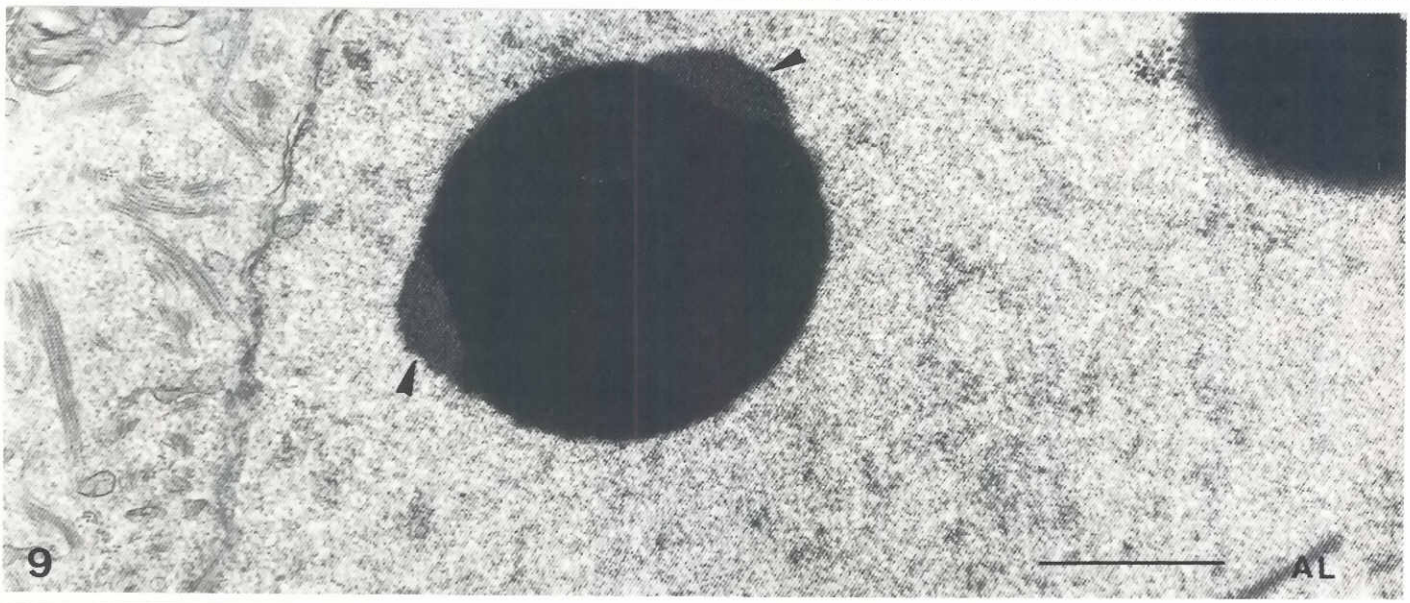
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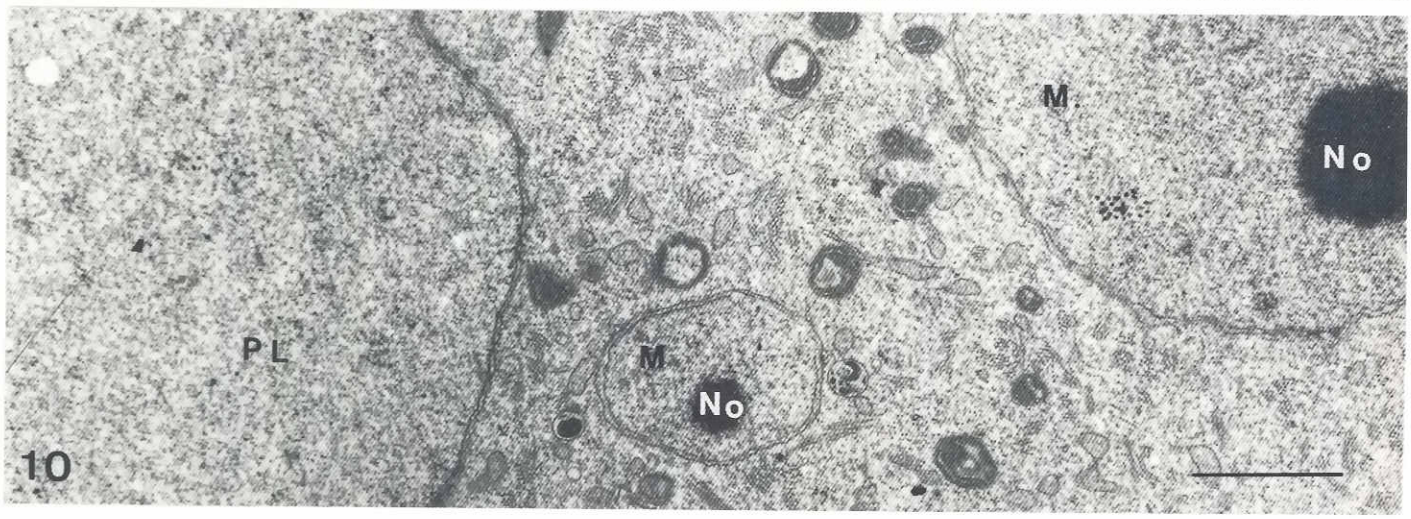
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1987; Benavente *et al.*, 1989). Similar events occurring during the completion of meiotic maturation in activated oocytes may be necessary for programming some pronuclear functions. Formation of a mature pronucleus may also require the active transport of proteins via the nuclear envelope: those supplied by an oocyte, and those which appear as new after egg activation (Dreyer, 1987; Howlett *et al.*, 1988).

In the present study we demonstrate that conditions promoting chromatin condensation of nuclei of 8-cell morulae are maintained by metaphase II mouse oocyte and disappear shortly after oocyte activation (Fig. 16). Passage of 8-cell nucleus through metaphase II cytoplasm brings about an additional effect — chromatin pulverization — typical for PCC of S-phase nucleus (Rao and Johnson, 1974; Heneen and Röhme, 1982). In the fourth cleavage cycle of a mouse embryo, the S-phase is the longest phase (7 out of 10-12 h) whereas G1 and G2 phases last about 1-2 h each (Smith and Johnson, 1986). Blastomere chromatin condenses in a pulverized manner, but even the most condensed fragments of chromatin do not approach the level of condensation of meiotic chromosomes. Other effects of PCC are: formation of an incomplete spindle, absence of mature kinetochores on condensed chromosomes and dispersion of chromatin. Full chromosome condensation and formation of the mature kinetochore are possible only when a donor nucleus derives from a G1 or G2 cell (Zinkowski *et al.*, 1991; Collas *et al.*, 1992b).

Further we have shown that an 8-cell nucleus passaged through metaphase II oocyte and induced to PCC transforms into pronucleus-like nucleus through the stages equivalent to those observed in meiotic chromatin of activated oocyte (anaphase, telophase) (Fig. 16). Because the blastomere chromatin is less condensed than the oocyte chromatin, it can reform interphase nucleus (i) ahead of the egg pronucleus. The «pronucleus» formed from 8-cell chromatin is in many respects similar to the egg pronucleus. The remodeling process is most spectacular in the case of nucleoli which revert from a morphology characteristic for a transcriptionally active embryonic cell (Szöllösi, 1971) to a structure typical for the egg cell not engaged in rRNA transcription. Also the appearance of blebs in the nuclear envelope, the activity which is normally extinguished in mouse embryos following activation of the embryonic genome (Szöllösi and Szöllösi, 1988) may be taken as evidence of functional reprogramming. However, such a «pronucleus» is constituted of mosaic chromatin composed of replicated and non-replicated DNA segments, and it is unknown whether it can resume DNA synthesis in the first cell cycle. Recent experiments in the rabbit have shown that transfer of embryonic late S-phase nuclei to enucleated oocytes affects the developmental potential of transplant embryos suffering from multiple chromosome abnormalities (Collas *et al.*, 1992a,b).

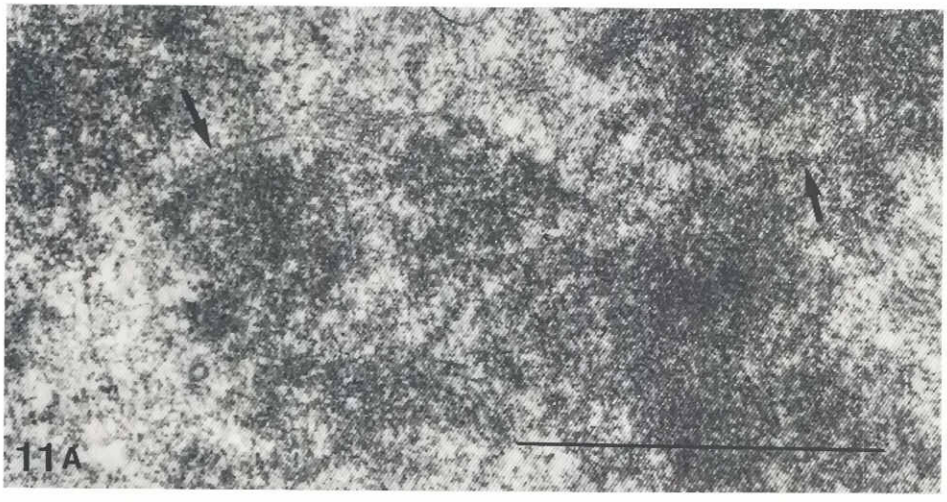
We have also found that the reactivity of a blastomere nucleus to the ooplasmic factors changes rapidly after activation of the mouse oocyte. Eight-cell nuclei transferred within a narrow 30-40 min window after activation could either pass PCC (and be thus synchronized with oocyte chromatin) or could remain in interphase (Fig. 16). This shows that the blastomere nucleus becomes resistant to the declining activity of MPF at metaphase-interphase transition. In our previous experiments with fusion of thymocytes, the time interval permitting PCC was estimated at up to 1-1.5 h, that is to the end of meiotic telophase II. Eight-cell nucleus may be less sensitive to MPF due to its larger size, organization of nuclear lamina, and/or contribution of euchromatin, which is particularly abundant in the nuclei of early cleavage embryos. Limited mixing of cytoplasms, noted by us in hybrids constructed from activated eggs, may also slow down the access of ooplasmic factors to the blastomere nucleus.

Enucleated metaphase II oocytes have been used as recipients for embryonic nuclei in several species of mammals, and reconstituted embryos developed up to term (reviewed by First and Prather, 1991). However in the mouse, most of the transplant embryos did not develop beyond the first cleavages (Tsunoda *et al.*, 1989; Waksmundzka, personal communication). Aberrant segregation of chromosomes during the first mitoses (Waksmundzka, personal communication) brought about by the nucleus/cytoplasm asynchrony may indeed be an important, but it is not the only cause of restricted development of these embryos. We believe that the capability of mouse blastomere nuclei of undergoing remodeling and reprogramming in oocyte cytoplasm is limited mainly because the blastomere-specific nuclear proteins cannot be replaced easily by the egg-specific proteins. Arguments supporting our opinion are the following. 1) Nuclear transfer experiments by Waksmundzka (personal communication) have shown that even in the case of perfect synchronization of the nucleus/cytoplasm cycles (4-cell metaphase chromosomes to metaphase II cytoplasm), transplant embryos do not develop beyond the morula stage. This shows that mitotic chromosomes of an embryonic cell are not equivalent to meiotic chromosomes of an oocyte. 2) Eight-cell nucleus induced to PCC in metaphase II oocyte does not usually condense to a degree comparable with meiotic chromatin (mouse: this work, rabbit: Collas *et al.*, 1992b) or PCC of G1 thymocyte (Szöllösi *et al.*, 1986a). 3) Recent experiments of Kono *et al.* (1991) provide evidence that 1/8 nucleus cannot be substantially reprogrammed in meiotic cytoplast produced from telophase I oocyte and «matured» *in vitro* for a few hours. Transplant embryos receiving the nucleus rarely developed to 8-cell embryos, and only occasionally into blastocyst. All these data taken together show that among potential donors for cloning experiments in the mouse, 8-cell blastomeres may belong to the worst. Reprogramming an 8-cell nucleus may

Fig. 8. Condensation of S-phase blastomere nucleus. (A) *PCC from the same oocyte as shown in Fig. 3 at higher magnification. The microtubules (Mt) are well oriented along a spindle axis. Note the more condensed regions of the chromosomes (arrows). Bar: 1 μm.* **(B)** *Different levels of condensation are reached in different portions of chromatin. Microtubules are present in the proximity of the chromatin. Bar: 1 μm.*

Fig. 9. Prematurely condensed blastomere nucleus transforms into a pronucleus-like nucleus after oocyte activation. *The chromatin is dispersed, the nucleoli are composed of densely packed filaments. Two lenticular bodies on the surface of nucleolus (arrowheads) correspond to the nucleolar organizer regions (NORs). NORs are present also on the surface of other nucleoli (not shown). Note the occurrence of annulate lamellae (AL) characteristic for blastomere nuclei, but rare in the egg pronuclei. Bar: 1 μm.*

Fig. 10. Another example of pronucleus-like nucleus (PL) reformed from PC-blastomere nucleus in an activated oocyte. *Four hours culture post activation. Several micronuclei (M) are present, containing one or more nucleoli (No). Bar: 1 μm.*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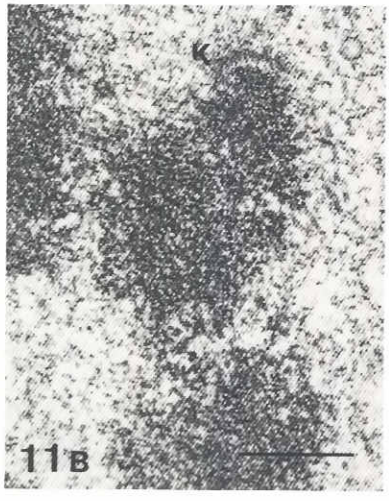
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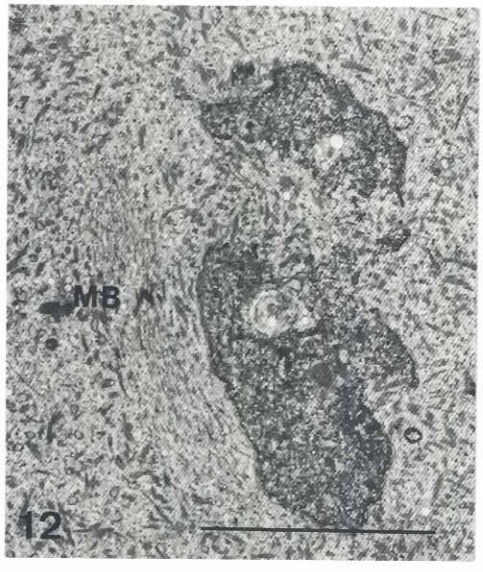
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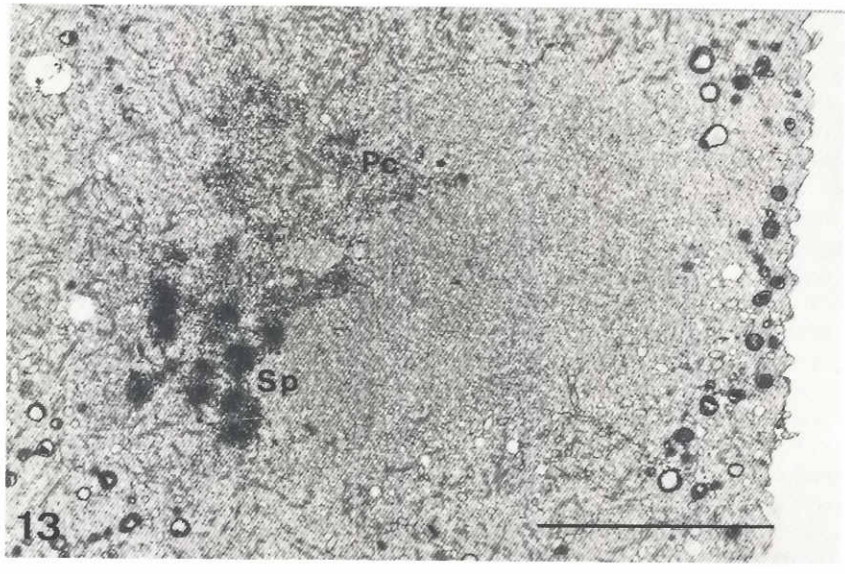
Pc
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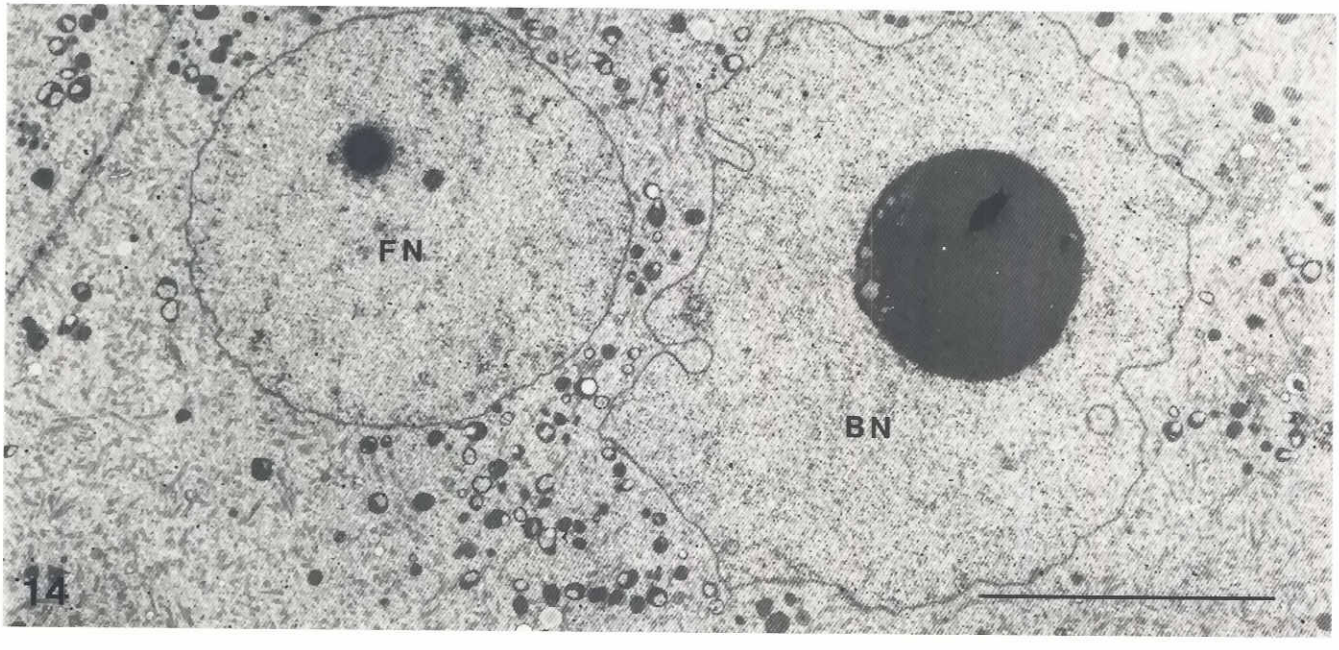
13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14

appear impossible, at least using standard conditions for nuclear transplantation.

In contrast to the deep reorganization of a blastomere nucleus in metaphase II oocyte, only minor modifications of a nucleus occur in the interphase egg. We have observed that nuclei introduced 1 h or later after egg activation retained interphase 8-cell morphology (Fig. 16). The only changes were: blebbing of the nuclear envelope (thus this activity is apparently independent of previous PCC) and nuclear swelling. Transferred nucleus competes with the resident egg pronucleus suppressing its growth (Czolowska *et al.*, 1984; Kubiak *et al.*, 1991; this paper). This shows that the pool of karyophilic materials in the cytoplasm is limited, and that these materials are taken preferentially by a nucleus more advanced in interphase. The «pool» must consist mostly of oocyte-made compounds, such as snRNAs and sRNPs (Dean *et al.*, 1989) and laminas (Kubiak *et al.*, 1991) because it is exhausted within a few hours after egg activation. Some karyophilic proteins moving via an active transport may be similar to the «early shifting proteins» which accumulate in embryonic nuclei of *Xenopus* (Dreyer, 1987).

In numerous experiments using 1-cell mouse embryos as recipients for 8-cell nuclei (reviewed by Prather and First, 1990), the eggs were manipulated (enucleation and fusion) following formation of large visible pronuclei (zygotes) and not earlier than 4-6 h after activation (parthenotes) (McGrath and Solter, 1984; Surani *et al.*, 1986; Barnes *et al.*, 1987; Howlett *et al.*, 1987). Thus these manipulations were done outside the window which permits PCC, and reconstituted embryos initiated development with an intact 8-cell nucleus. Most of the pronuclear-specific proteins made during oogenesis must have been removed with aspirated pronuclei. In consequence, the developmental potential of these egg cells was abruptly restricted beginning with the first cycle (90% of transplant eggs of mouse carrying 8-16 cell nuclei block at 1- and 2-cell stages, and blastocysts form only sporadically; McGrath and Solter, 1986; Howlett *et al.*, 1987), though partial reprogramming of an 8-cell nucleus must have occurred, as evidenced by expression of 70 kD proteins at a scheduled time (Barnes *et al.*, 1987; Howlett *et al.*, 1987).

The experimental system employed in our work differs from classic experiments because we employed nucleated oocytes and eggs instead of anucleate cytoplasts as recipients of blastomere nuclei. We believe, however, that nuclear changes evoked in hybrid cells are similar to processes occurring in enucleated oocytes, although the absence of the native nucleus in the latter cells may influence the extent and/or the dynamics of changes in the transferred nucleus. For instance, the extent of chromatin remodeling in blastomere nuclei introduced into metaphase II cytoplasts is probably decreased due to the disappearance of PCC-promoting activity in enucleated oocytes (Czolowska *et al.*, 1986). In contrast, it is very likely that in enucleated, recently activated interphase egg cytoplasts, the uptake of pronucleus-specific proteins by blastomere nuclei is enhanced due to elimination of a competing egg pronucleus. However, as was demonstrated in multiple experiments (as we have seen earlier) development of nuclear transplant mouse eggs carrying the blastomere nucleus from the 8-cell stage invariably stops. We believe that true functional reprogramming of blastomere nucleus in the mouse egg (irrespective of whether it is introduced into metaphase II oocytes or pronuclear eggs) never occurs because: 1) one-cell egg does not provide the quantity of factors necessary for nuclear remodeling; 2) nuclear envelope breakdown and chromatin condensation in metaphase II oocytes are not followed by total displacement of blastomere-specific nuclear proteins; 3) condensation of S-phase blastomere nucleus in metaphase II oocyte may be followed by chromosome anomalies; 4) the transferred 8-cell nucleus cannot eventually complete remodeling at following mitotic divisions (as discussed above) because there is only one cleavage division separating the 2-cell stage within which activation of the embryonic genome should occur; 5) the transferred 8-cell nucleus may enter the second cell cycle of the recipient embryo as a mosaic of reprogrammed and non-reprogrammed chromatin segments (or genes).

Materials and Methods

Collection and activation of oocytes

Ovulated metaphase II oocytes were obtained from randomly bred (Swiss albino), inbred (CBA/H) and F1 (C57BL/10x CBA) female mice induced to ovulate with PMSG (Pregnant Mare's Serum Gonadotrophin) and hCG (human Chorionic Gonadotrophin) (doses: 5-10 IU of each given 48-56 h apart). Oocytes were harvested 12-18.5 h after hCG injection, treated with hyaluronidase (150-300 IU/ml) for removal of cumulus cells, and incubated in 0.5% pronase for zona pellucida digestion either before or after artificial activation. Short-term culture of oocytes in drops of M2 culture medium (Fulton and Whittingham, 1978) under paraffin oil, at 37°C, was followed by fusion (see below) or activation. Parthenogenetic activation (oocyte age 16-19.5 h after hCG) was achieved by treating the oocytes with 8% ethanol for 4 to 6 min (Cuthbertson *et al.*, 1981; Cuthbertson, 1983) in drops of the activating medium under paraffin oil.

Blastomeres

Cleaving 6- to 8-cell embryos were obtained on the third day after finding a copulation plug from Swiss albino, CBA/H and F1 females mated to Swiss or F1 males. Spontaneously ovulating females (autopsied between 9:30 and 12 a.m.) and superovulated females (autopsied 62-68 h after hCG injection) were used as donors of embryos. Embryos were treated with 0.5%

Fig. 11. Oocytes fused with a blastomere shortly before activation. (A) *PCC of 1/8 nucleus has occurred. Chromatin is unevenly condensed and only immature kinetochores (arrows), composed of a single dense plaque develop. Bar: 1.0 μm.* **(B)** *On rare occasions, blastomere chromatin condenses as highly as meiotic chromosomes, and mature kinetochores (K) develop. This shows that the donor blastomere nucleus must have been at the end of G2. Bar: 0.35 μm.*

Fig. 12. Oocyte fused with a blastomere just before activation. *PCC and NE breakdown was apparently initiated, but not completed. Cytoplasmic components penetrate freely between the chromatin islands. Parallel to the nucleus is a small telophase spindle which develops synchronously with meiotic telophase II spindle, but lacks chromosomes and MTOCs. A small midbody (MB) is present, however. Bar: 10 μm.*

Fig. 13. Oocyte fused with a blastomere shortly after activation. *Highly condensed oocyte chromosomes are in anaphase. Associated with them is a large island of pulverized 1/8 chromatin (Pc). Sp: spindle microtubules. Bar: 10 μm.*

Fig. 14. When the fusion of a blastomere is performed 1 h or later after oocyte activation, the blastomere nucleus (BN) does not change at all during forthcoming hours of culture. *Its NE remains folded and the nucleolus is bipartite, as in the control blastomere nucleus (compare Fig. 2A, B). The female pronucleus (FN), in contrast, has a smooth NE and contains a compact nucleolus. Bar: 10 μm.*

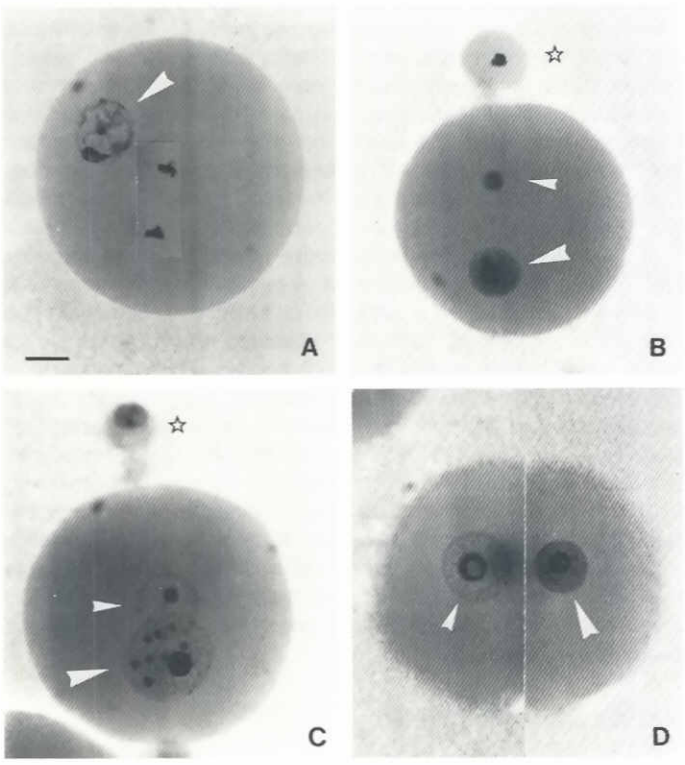

Fig. 15. Morphology of blastomere nuclei (long arrowhead) introduced into activated oocytes. (A) *1/8 nucleus transferred to oocyte 40 min after activation is slightly changed, but retains interphase character, whereas oocyte chromatin completes the second meiotic division (1 h culture post fusion). 1/8 nuclei introduced into oocytes 1 h after activation remain intact during the first hour of culture* **(B)**, *and undergo swelling during subsequent hours* **(C)**. *1/8 nucleus introduced 4-5 h after activation remains intact during subsequent 4 h of culture* **(D)**. *Short arrowhead points to the position of the egg pronucleus. Asterisk marks the second polar body. Bar: 10 μm.*

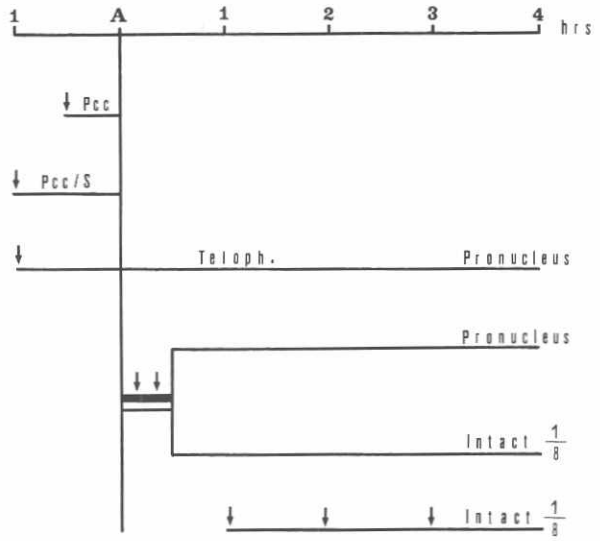

Fig. 16. Reaction of blastomere (1/8) nucleus to ooplasmic factors depending on the sequence of fusion (arrow) and activation (A), and the duration of culture (in h). *In a non-activated oocyte, premature chromosome condensation (PCC) of a nucleus occurs within 30 min, and after ... a spindle (S) is induced. After oocyte activation condensed chromatin becomes synchronized with oocyte chromatin (telophase) and reforms an interphase nucleus (i) of the pronuclear type. Nuclei introduced into oocytes close to the activation time (black and white bar) can be induced to undergo PCC and follow the route of the pronuclear development, or can remain in interphase. 1/8 nucleus introduced into an egg cell 1 h or later after activation remains intact.*

pronase and disaggregated in calcium- and magnesium-free Dulbecco's saline, or in 0.02% EDTA in Dulbecco's saline with addition of bovine serum albumin (BSA). Isolated blastomeres that have entered the fourth cell cycle (= 1/8 blastomeres) were stored in the culture medium before further manipulation.

Fusion

Metaphase II oocytes or previously activated oocytes were agglutinated with single 1/8 blastomeres using phytohemagglutinin (PHA; 150 or 300 μg/ml BSA-free M2 medium). Oocytes and blastomeres were first incubated for a few minutes in PHA and then squeezed in a pipette to increase contact between cells. Oocytes with an attached blastomere were immersed for 30-60 sec in polyethylene glycol (PEG; Fluka, Mr 2000, 50% w/v in BSA-free M2 medium), thoroughly washed afterwards, placed in separate drops of culture medium and cultured under standard conditions. Fusion of an oocyte with a blastomere was monitored *in vitro*.

Light and electron microscopy

For light microscopy, hybrid cells were prepared as whole mounts using the method of Tarkowski and Wróblewska (1967). For electron microscopy, material was processed as described previously (Szöllösi *et al.*, 1986a, 1988).

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References

ALBERTS, B., BRAY, D., LEWIS, J., RAFF, M., ROBERTS, K. and WATSON, J.D. (1989). The cell nucleus. In *Molecular Biology of the Cell*. Garland Publishing, Inc., New York, London, pp. 481-549.

BARNES, F.L., ROBL, J.M. and FIRST, N.L. (1987). Nuclear transplantation in mouse embryos: assessment of nuclear function. *Biol. Reprod. 36:* 1267-1274.

BENAVENTE, R., SCHEER, U. and CHALY, N. (1989). Nucleocytoplasmic sorting of macromolecules following mitosis: fate of nuclear constituents after inhibition of pore complex function. *Eur. J. Cell Biol. 50:* 209-219.

COLLAS, P. and ROBL, J.M. (1991). Relationship between nuclear remodeling and development in nuclear transplant rabbit embryos. *Biol. Reprod. 45:* 455-465.

COLLAS, P., BALISE, J.J. and ROBL, J.M. (1992a). Influence of cell cycle stage of the donor nucleus on development of nuclear transplant rabbit embryos. *Biol. Reprod. 46:* 492-500.

COLLAS, P., PINTO-CORREIA, C., PONCE DE LEON, F.A. and ROBL, J.M. (1992b). Effect of donor cell cycle stage on chromatin and spindle morphology in nuclear transplant rabbit embryos. *Biol. Reprod. 46:* 501-511.

CUTHBERTSON, K.S.R. (1983). Parthenogenetic activation of mouse oocytes with ethanol and benzyl alcohol. *J. Exp. Zool. 226:* 311-314.

CUTHBERTSON, K.S.R., WHITTINGHAM, D.G. and COBBOLD, P.H. (1981). Free Ca^{2+} increases in exponential phases during mouse oocyte activation. *Nature 294:* 754-757.

CZOLOWSKA, R., MODLINSKI, J.A. and TARKOWSKI, A.K. (1984). Behaviour of thymocyte nuclei in non-activated and activated mouse oocytes. *J. Cell Sci. 69:* 19-34.

CZOLOWSKA, R., WAKSMUNDZKA, M., KUBIAK, J.Z. and TARKOWSKI, A.K. (1986). Chromosome condensation activity in ovulated metaphase II mouse oocytes assayed by fusion with interphase blastomeres. *J. Cell Sci. 84:* 129-138.

DEAN, W.L., SEUFERT, A.C., SCHULTZ, G.A., PRATHER, R.S., SIMERLY, C., SCHATTEN, G., PILCH, D.R. and MARZLUFF, W.F. (1989). The small nuclear RNAs for pre-mRNA splicing are coordinately regulated during oocyte maturation and early embryogenesis in the mouse. *Development 106:* 325-334.

DERENZINI, M., THIRY, M. and GOESSENS, G. (1990). Ultrastructural cytochemistry of the mammalian cell nucleolus. *J. Histochem. Cytochem. 38:* 1237-1256.

DiBERARDINO, M.A. (1988). Genomic multipotentiality of differentiated somatic cells. In *Regulatory Mechanisms in Developmental Processes* (Eds. G. Eguchi, T.S. Okada and L. Saxén). Elsevier Scientific Publishers, Ireland, pp. 129-136.

DREYER, C. (1987). Differential accumulation of oocyte nuclear proteins by embryonic nuclei of *Xenopus*. *Development 101:* 829-846.

DYBAN, A.P., LEE, K., O'NEILL, G.T., SPEIRS, S. and KAUFMAN, M.H. (1988). Cytogenetic study of silver-staining NOR in 8-cell-stage mouse blastomeres fused to 1-cell-stage embryos. *Development 104:* 453-463.

FAKAN, S. and HERNANDEZ-VERDUN, D. (1986). The nucleolus and the nucleolar organizer regions. *Biol. Cell 56:* 189-206.

FIRST, N.L. and PRATHER, R.S. (1991). Genomic potential in mammals. *Differentiation 48:* 1-8.

FULTON, B.P. and WHITTINGHAM, D.G. (1978). Activation of mammalian oocytes by intracellular injection of calcium. *Nature 273:* 149-151.

HASHIMOTO, N. and KISHIMOTO, T. (1988). Regulation of meiotic metaphase by a cytoplasmic maturation-promoting factor during mouse oocyte maturation. *Dev. Biol. 126:* 242-252.

HENEEN, W.K. and RÖHME, D. (1982). Spindle formation and chromosome segregation associated with premature chromosome condensation. In *Premature Chromosome Condensation: Applications in Basic, Clinical and Mutation Research* (Eds. P.T. Rao, R.T. Johnson and K. Sperling). Academic Press, New York, pp. 79-97.

HOWLETT, S.K., BARTON, S.C., NORRIS, M.L. and SURANI, M.A.H. (1988). Nuclear and cytoplasmic localization of newly synthesized proteins in the early mouse embryo. *Development 103:* 129-134.

HOWLETT, S.K., BARTON, S.C. and SURANI, M.A. (1987). Nuclear cytoplasmic interactions following nuclear transplantation in mouse embryos. *Development 101:* 915-923.

KELLENBERGER, E. (1987). The compactness of cellular plasmas; in particular, chromatin compactness in relation to function. *Trends Biochem. Sci. 12:* 105-107.

KONO, T., KWON, O.Y. and NAKAHARA, T. (1991). Development of enucleated mouse oocytes reconstituted with embryonic nuclei. *J. Reprod. Fertil. 93:* 165-172.

KUBIAK, J.Z., PRATHER, R.S., MAUL, G.G. and SCHATTEN, G. (1991). Cytoplasmic modification of the nuclear lamina during pronuclear-like transformation of mouse blastomere nuclei. *Mech. Dev. 35:* 103-111.

MASUI, Y. (1985). Problems of oocyte maturation and the control of chromosome cycles. *Dev. Growth Differ. 27:* 295-309.

McGRATH, J. and SOLTER, D. (1984). Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development *in vitro*. *Science 226:* 1317-1319.

McGRATH, J. and SOLTER, D. (1986). Nucleocytoplasmic interactions in the mouse embryo. *J. Embryol. Exp. Morphol. 97 (Suppl.):* 277-289.

NAGL, W. (1985). Chromatin organization and the control of gene activity. *Int. Rev. Cytol. 94:* 21-56.

PRATHER, R.S. and FIRST, N.L. (1990). Nuclear transfer in mammalian embryos. *Int. Rev. Cytol. 120:* 169-190.

RAO, P.N. and JOHNSON, R.T. (1974). Regulation of cell cycle in hybrid cells. In *Control of Proliferation in Animal Cells*. Cold Spring Harbor Conference on Cell Proliferation (Eds. B. Clarkson and R. Baserga), Vol. 1. Cold Spring Harbor Lab., New York, pp. 785-800.

SMITH, R.K.W. and JOHNSON, M.H. (1986). Analysis of the third and fourth cell cycles of mouse early development. *J. Reprod. Fertil. 76:* 393-399.

SORENSEN, R.A., CYERT, M.S. and PEDERSEN, R.A. (1985). Active maturation-promoting factor is present in mature mouse oocytes. *J. Cell Biol. 100:* 1637-1640.

SURANI, M.A.H., BARTON, S.C. and NORRIS, M.L. (1986). Nuclear transplantation in the mouse: heritable differences between parental genomes after activation of the embryonic genome. *Cell 45:* 127- 136.

SWANSON, J.A. and McNEIL, P.L. (1987). Nuclear reassembly excludes large molecules. *Science 238:* 548-550.

SZÖLLÖSI, D. (1971). Nucleoli and ribonucleoprotein particles in the preimplantation conceptus of the rat and the mouse. In *The Biology of the Blastocyst* (Ed. R.J. Blandau). The University of Chicago Press, Chicago, London, pp. 95-113.

SZÖLLÖSI, D., CZOLOWSKA, R., SOLTYNSKA M.S. and TARKOWSKI, A.K. (1986a). Ultrastructure of cell fusion and premature chromosome condensation (PCC) of thymocyte nuclei in metaphase II mouse oocytes. *Biol. Cell 56:* 239-250.

SZÖLLÖSI, D., CZOLOWSKA, R., SZÖLLÖSI, M.S. and TARKOWSKI, A.K. (1986b). Remodeling of thymocyte nuclei in activated mouse oocytes: an ultrastructural study. *Eur. J. Cell Biol. 42:* 140-151.

SZÖLLÖSI, D., CZOLOWSKA, R., SZÖLLÖSI, M.S. and TARKOWSKI, A.K. (1988). Remodeling of mouse thymocyte nuclei depends on the time of their transfer into activated, homologous oocytes. *J. Cell Sci. 91:* 603-613.

SZÖLLÖSI, M.S. and SZÖLLÖSI, D. (1988). «Blebbing» of the nuclear envelope of mouse zygotes, early embryos and hybrid cells. *J. Cell Sci. 91:* 257-267.

TARKOWSKI, A.K. and WRÓBLEWSKA, J. (1967). Development of blastomeres of mouse eggs isolated at 4- and 8-cell stages. *J. Embryol. Exp. Morphol. 18:* 155-180.

TSUNODA, Y., TOKUNAGA, T., IMAI, H. and UCHIDA, T. (1989). Nuclear transplantation of male primordial germ cells in the mouse. *Development 107:* 407-411.

TSUNODA, Y., YASUI, T., SHIODA, Y., NAKAMURA, K., UCHIDA, T. and SUGIE, T. (1987). Full-term development of mouse blastomere nuclei transplanted into enucleated two-cell embryos. *J. Exp. Zool. 242:* 147-151.

WEBER, M., KUBIAK, J.Z., ARLINGHAUS, R.B., PINES, J. and MARO, B. (1991). C-mos proto-oncogene product is partly degraded after release from meiotic arrest and persists during interphase in mouse zygotes. *Dev. Biol. 148:* 393-397.

ZINKOWSKI, R.P., MEYNE, J. and BRINKLEY, B.R. (1991). The centromere-kinetochore complex: a repeat subunit model. *J. Cell Biol. 113:* 1091-1110.

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