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Developmental regulation of chromatin composition during mouse embryogenesis: somatic histone H1 is first detectable at the 4-cell stage

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Summary

We have examined the distribution of histone H1 in oocytes and preimplantation embryos of the mouse, using a polyclonal antibody raised against the histone H1 subtypes present in somatic cells. Immunofluorescence and immunoblotting analyses failed to detect somatic histone H1 in germinal vesicle (GV)-stage oocytes. In contrast, somatic histone H1 was detectable by immunofluorescence in the nuclei of GV oocytes previously injected with histone H1 as well as the nuclei of ovarian granulosa cells, and by immunoblotting in 8-cell embryos. 1- and 2-cell embryos examined by immuofluorescence did not contain detectable somatic histone H1. At the early 4-cell stage (54-56 hours post-hCG), 5 of 52 embryos contained somatic histone H1 in one or more nuclei. By the late 4-cell stage (66-68 hours post-hCG), however, 58 of 62 embryos contained somatic histone H1. In 8-cell embryos, morulae and blastocysts, all nuclei contained somatic histone H1 in every case. When embryos were exposed to the transcriptional inhibitor, α-amanitin, beginning at the late 2-cell stage, they cleaved to the 4-cell stage but fewer than 10% developed histone H1 immunoreactivity. When treatment began at the early 4-cell stage, the embryos that remained at the 4-cell stage in the presence of the drug developed histone H1 immunoreactivity in half of the cases. Embryos that reached the 5- to 8-cell stage in the presence of the drug developed histone H1 immunoreactivity in every case. The protein synthesis inhibitor, puromycin, prevented development of histone H1 immunoreactivity in most embryos when added either at the late 2-cell or early 4-cell stage. When embryos were exposed to the DNA replication inhibitor, aphidicolin, beginning at the late 2-cell stage, they cleaved to the 4-cell stage, but developed only a very weak histone H1 immunoreactivity. These results indicate that oocytes and 1- and 2-cell embryos contain little or no somatic histone H1, which may imply that these cells contain immunologically distinct histone H1 subtypes. The somatic subtypes first appear at the 4-cell stage, through a process requiring embryonic transcription and DNA replication during the third cell cycle. These results suggest that the deposition of somatic histone H1 on chromatin is developmentally regulated during mouse embryogenesis.

Key words: Histone H1, mouse, oocyte, embryogenesis, chromatin.

Introduction

It is well established that extensive changes in nuclear activity occur during early mammalian embryogenesis. For instance, newly fertilized embryos are transcriptionally inactive, and transcription begins at a species-specific stage during early cleavage (reviewed by Telford et al., 1990). Nuclear transplantation experiments have revealed that nuclei of early 2-cell mouse embryos can direct normal embryonic development when transplanted into enucleated 1-cell embryos, whereas nuclei obtained from more advanced embryos are rarely able to do so (McGrath and Solter, 1984; Robl et al., 1986; Howlett et al., 1987). Analyses of DNA methylation in mouse embryos have indicated that there is a decline in the degree of methylation of certain sequences between the 1-cell and blastocyst stages (Howlett and Reik, 1991; Monk et al., 1991). It also has been observed that plasmid-borne genes can be expressed independently of enhancer elements when they are microinjected into 1-cell mouse embryos, whereas enhancers are required when the plasmids are introduced into 2-cell embryos (Wiekowski et al., 1991). These alterations in nuclear properties suggest that underlying changes in chromatin composition may occur during early development.

As key structural components of chromatin, the histones, and histone H1 in particular, may play central roles in such changes. Most eukaryotes contain several histone H1 subtypes encoded by different genes (Lennox and Cohen, 1984; review: van Holde, 1989). During early embryogenesis of many non-mammalian species, different subtypes become

associated with chromatin at specific stages of development (reviewed by Poccia, 1986). Sea urchin oocytes contain a subtype known as cleavage-stage (cs) H1. Following fertilization, csH1 continues to be synthesized using maternal transcripts until about the 8- to 16-cell stage, when there appears a second subtype, αH1, that dilutes out the csH1. The αH1 itself is later replaced by embryonically encoded βH1 and γH1, which remain in adult animals. Two major histone H1 subtypes have been identified in the sea worm (Franks and Davis, 1983). Histone H1m is present in oocytes and remains detectable up to the blastula stage. A presumably embryo-encoded subtype, H1e, becomes predominant during late cleavage stages. Similarly, there exists in *Xenopus* an H1-like protein that is present only in eggs and early embryos (Smith et al., 1988). In the mud snail, the histone H1 subtype present in oocytes continues to be synthesized in embryos until about the 32- to 64-cell stage (Flenniken and Newrock, 1987). As in the sea urchin, several embryonic subtypes are then transiently produced until the adult profile is established. These results indicate that a developmentally regulated programme of histone H1 subtype switching occurs during early embryogenesis in these animals.

In the mouse, there is no information concerning histone H1 subtype switching during early embryogenesis, although changes in core histone mRNA populations during this time have been described (Giebelhaus et al., 1983; Graves et al., 1985). In the experiments reported here, we have used an antibody raised against the histone H1 subtypes present in somatic cells (Bustin and Stollar, 1973; Sluyser and Bustin, 1974) to examine the distribution of histone H1 in mouse oocytes and embryos. Our results show that somatic histone H1 is not detectable in oocytes or in early cleavage-stage embryos, and that it appears during the 4-cell stage and thereafter remains detectable throughout preimplantation development. This change in the histone composition of chromatin during early mouse embryogenesis could be related to the changes in nuclear activity that occur during this period.

Materials and methods

Collection of oocytes and embryos

To obtain immature oocytes arrested at prophase I of meiosis, CD-1 female mice were killed and their ovaries were removed and transferred to Hepes-buffered minimum essential medium (MEM-H) modified as described (Schroeder and Eppig, 1984; Clarke et al., 1988) and supplemented with 50 μg/ml dibutyryl cyclic AMP (dbcAMP). The ovarian follicles were punctured using a 30-gauge needle to release the enclosed oocytes, and immature oocytes were recognized by the presence of the germinal vesicle (GV). The dbcAMP prevents the immature oocytes from undergoing germinal vesicle breakdown (GVBD) in culture (Cho et al., 1974). Immature oocytes were transferred to modified MEM containing 10% fetal calf serum (FCS) and 50 μg/ml dbcAMP, and cultured at 37°C in an atmosphere of 5% CO_2 in air for 2 hours prior to microinjection.

To obtain embryos, CD-1 female mice were superovulated by an injection of 5 i.u. pregnant mares' serum gonadotropin (Sigma) followed 44-48 hours later by 5 i.u. of human chorionic gonadotropin (hCG; Sigma), and placed with CD-1 males after the hCG injection. The next morning, the females were checked for the presence of a vaginal plug to confirm that mating had occurred. 1-cell embryos were recovered from the oviducts of females killed on day 0.5 (day 0 = day of plug). 2-cell embryos were obtained by flushing the oviducts of females killed on day 1.5. Older embryos were obtained by culturing embryos recovered at the 2-cell stage. In this case, the 2-cell embryos were transferred to CZB medium (Chatot et al., 1989) and incubated at 37°C in an atmosphere of 5% O_2, 5% CO_2, 90% N_2.

Microinjection of oocytes

Oocytes to be microinjected were placed in a 5 μl drop of MEM-H containing 50 μg/ml dbcAMP on the lid of a 35 mm Petri dish overlaid with paraffin oil. Microinjection was carried out using a Leitz Labovert FS inverted microscope and Leitz micromanipulators. The holding and injection pipettes, plastic tubing and glass syringes connected to the micromanipulators were filled with light paraffin oil. To control the volume of solution injected into each oocyte, an ocular micrometer was used to monitor the movement of the aqueous-oil interface during injection. Oocytes were injected with about 10 pl of a solution of 1 mg/ml histone H1 (Boehringer) in water. Microinjected oocytes were transferred to modified MEM containing 10% FCS and 50 μg/ml dbcAMP, and incubated overnight at 37°C in an atmosphere of 5% CO_2 in air.

Affinity-purification of antibody

The antibodies used in these experiments were obtained from rabbits immunized using rat thymus histone H1 and recognize the major histone H1 subtypes present in somatic cells (Bustin and Stollar, 1973; Sluyser and Bustin, 1974). For the present study, lyophilized antisera recognizing peaks II and V of rat histone H1 were reconstituted in aqueous medium. Anti-histone H1 antibodies were purified from the reconstituted serum by affinity-purification (Lasko and Ashburner, 1990; P. Lasko, personal communication). Affigel-10 beads (Bio-Rad) were incubated with poly-L-lysine (Sigma) or with calf thymus histone H1 (Boehringer) in 0.1 M Hepes-KOH (pH 7.5) for 1 hour, the supernatant was removed, and the beads were incubated in 0.2 M glycine in Hepes-KOH for 1 hour to block remaining protein-binding sites. The suspension of conjugated beads was washed in PBS containing 10 mM NaN_3 and stored at 4°C.

The beads were loaded into disposable 1 ml plastic syringes and equilibrated by washing with phosphate-buffered saline (PBS). Antibodies in the sera that bound non-specifically to the beads or to positively charged molecules were removed by passing the sera six times through the beads conjugated with poly-L-lysine. The flow-through from the sixth pass plus a following 4 ml PBS wash were collected and passed 6 times through the column containing the histone H1-conjugated beads. This column was then washed extensively with PBS. Bound anti-histone H1 antibodies were eluted by washing the column with 0.1 M glycine (pH 2.5) and then with 0.1 M triethylamine (pH 11.5) (Harlow and Lane, 1988), and immediately neutralized by addition of 1 M Tris (pH 7.4). The antibodies were concentrated using spin-dialysis membranes (Amicon Canada) and stored at −70°C. The purified antibodies reacted with histone H1 as assayed by immunoblotting. A weak reaction with core histones was observed when these were present in large quantity.

Immunocytochemistry

Oocytes and embryos were freed of the zona pellucida using acidified (pH 2.5) Tyrode's medium (Hogan et al., 1986), washed in MEM-H, and fixed for 15 minutes at room temperature in a freshly prepared solution of 4% paraformaldehyde in Ca^{2+}-/Mg^{2+}-free PBS containing 0.1% Triton X-100. The fixed cells were washed several times in PBS containing 0.1% BSA and then incubated for 1 hour at

room temperature in a blocking solution (PBS, 3% BSA, 10% goat serum, 10% horse serum, 0.1% Triton X-100). The cells were then transferred to affinity-purified anti-histone H1 antibodies diluted 1:100 in blocking solution and incubated overnight at 4ºC in a humidified chamber. The cells were then washed 3× 20 minutes in blocking solution, incubated in biotinylated goat anti-rabbit IgG (Vector Labs) diluted 1:100 in blocking solution for 1 hour, washed as before, and incubated in streptavidin conjugated to Texas Red (Gibco Canada) diluted 1:100 in PBS for 1 hour. The cells were then washed several times in PBS and the DNA was stained using DAPI or Hoechst 22358. To mount the cells for viewing, a drop of Moviol (Hoechst Canada) prepared as described (Harlow and Lane, 1988) was placed on a siliconized glass microscope slide and the cells were carefully pipetted into the drop. A glass cover slip was then placed on top of the drop of Moviol. The weight of the cover slip caused the drop to spread, flattening the cells. Cells were examined using a Leitz Laborlux S microscope equipped for epifluorescence with UV and rhodamine filter sets.

Immunoblotting

Calf thymus total histone was obtained from Boehringer. Oocytes and embryos were transferred into gel loading buffer (Harlow and Lane, 1988), heated to 90ºC for 10 minutes, and stored at −20ºC. The cell lysates thus obtained were electrophoresed in 12% polyacrylamide gels containing 0.1% SDS. Following electrophoresis, the cellular material was transferred to nitrocellulose (Schleicher and Schuell, pore-size 0.2 μm) for 2 hours at 70 V at 4ºC in a buffer of 25 mM glycine, 25 mM ethanolamine, 10% methanol, 0.1% SDS (pH 9.7), and the membrane was allowed to dry overnight. The membrane was incubated for 1 hour at room temperature in a blocking solution consisting of 5% skim milk in 10 mM Tris, 140 mM NaCl (pH 7.5) (TBS) and then for 2 hours at room temperature in the primary antibody diluted 1:200 in blocking solution. Following 3 washes of 10 minutes each in 0.1% Tween-20, TBS, the membrane was incubated for 1 hour at room temperature in biotinylated donkey anti-rabbit IgG (Jackson Research Labs) diluted 1:500 in 3% BSA, 0.1% Tween-20, TBS. The membrane was washed as above, incubated for 1 hour at room temperature in streptavidin/alkaline phosphatase (Jackson Immunoresearch Labs) diluted 1:500 in 3% BSA, 0.1% Tween-20, TBS, and washed as above. Alkaline phosphatase activity was detected by incubation with BCIP and NBT (Harlow and Lane, 1988).

Drug treatments

Stock solutions of α-amanitin and aphidicolin (both from Boehringer) were prepared in water at a concentration of 10 mg/ml. Working solutions of 100 μg/ml were prepared by appropriate dilution of the stock into CZB medium. Puromycin (Sigma) was prepared as a stock solution of 1 mg/ml and used at a working strength of 10 μg/ml. Stock solutions were kept at −70ºC.

Results

Absence of detectable somatic histone H1 in the nuclei of immature oocytes

The first experiments were designed to determine whether histone H1 could be detected by immunofluorescence in the nuclei of immature oocytes. Oocytes containing a GV were isolated from ovarian follicles, fixed and reacted with the anti-histone H1 antibody. The oocytes were counter-stained with a fluorescent DNA-binding dye to allow the nuclei to be identified within the cell. In some cases, ovarian granulosa cells adhered to the oocytes, which permitted the distribution of histone H1 to be compared between the somatic and germ cells. In the granulosa cells, a bright fluorescence was observed that was restricted to the nuclei (Fig. 1A). In contrast, no fluorescence was observed in the oocyte nuclei (Fig. 1A, Table 1).

Presence of somatic histone H1 in immature oocytes injected with histone H1

To test whether the anti-histone H1 antibody could gain access to the nucleus of the oocyte, histone H1 was microinjected into immature oocytes. Microinjected histone H1 is transported to and accumulates in the nucleus of several

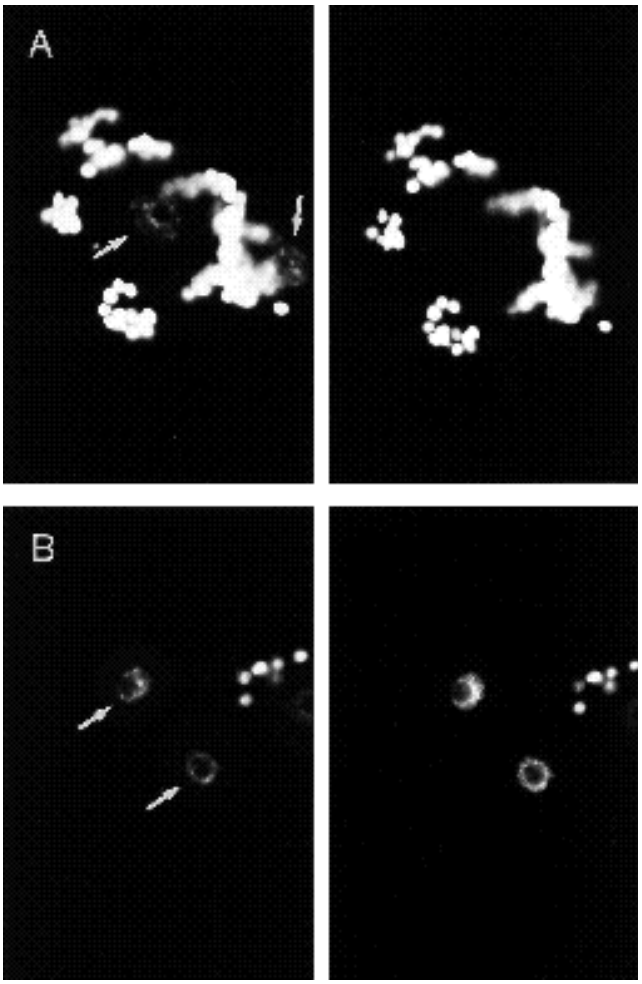


Fig. 1. Immunofluorescent staining of GV-stage oocytes not injected (A) or injected (B) with histone H1. (A) The left panel shows the location of the DNA revealed by Hoechst 33258 fluorescence. Arrows indicate two oocyte nuclei. Several ovarian granulosa cell nuclei are also visible. The right panel shows histone H1 revealed using Texas Red. The granulosa cell nuclei are fluorescent, but the oocyte nuclei are not. (B) Left: Hoechst. Right: Texas Red. Arrows indicate two oocyte nuclei. Microinjected histone H1 is detectable in each oocyte nucleus. The slight difference in apparent nuclear diameter between A and B is due to the fact that the oocytes become flattened to different extents when they are mounted onto slides.

Table 1. *The development of histone H1 immunoreactivity during mouse embryogenesis*

Time fixed (hours post-hCG)	Embryo stage	Number of embryos	+*	–
–	Immature oocyte	12	0	12
24	1-cell	10	0	10
48-54	2-cell	16	0	16
54-56	4-cell	52	5	47
66-68	4-cell	62	58	4
66-68	5- to 8-cell	37	37	0
70-74	5- to 8-cell	15	15	0
>90	Morula, blastocyst	25	25	0

*Embryos containing one or more fluorescent nuclei were scored as positive.

types including amphibian oocytes (Bonner, 1975; Breeuwer and Goldfarb, 1990). Following microinjection, the oocytes were incubated overnight in the presence of dbcAMP to prevent GVBD. Of 27 injected oocytes, 26 survived after overnight culture and 20 of these retained the GV. When these were reacted with the anti-histone H1 antibody, 19 of the 20 oocytes displayed a bright nuclear fluorescence as shown in Fig. 1B. This result demonstrated that the microinjected histone H1 accumulated in the nucleus and that it could be detected by immunofluorescence in this location. Therefore, the absence of nuclear fluorescence in uninjected oocytes reacted with the antibody was not due to an inability of the antibody to gain access to the nucleus.

Absence of somatic histone H1 in oocytes as assayed by immunoblotting

As an independent test of whether somatic histone H1 was present in oocytes, lysates were prepared and analyzed by immunoblotting. Using this antibody, the quantity of histone H1 present in 35 ng of calf thymus total histone is detectable (not shown). Lysates were prepared from 2820 metaphase II oocytes and 1480 late 4-cell or early 8-cell embryos. One oocyte is estimated to contain approximately 50 pg of histone (Wassarman and Mrozak, 1981; Schultz, 1986), so the histone present in the oocyte lysate was about 140 ng. Mouse diploid cells contain 6 pg of histone, so the embryo lysate contained about 70 ng of total histone. Thus, the oocyte lysate contained about twice as much histone as the embryo lysate. The lysates were loaded onto the same gel, subjected to SDS-PAGE, blotted onto nitrocellulose paper, and reacted with the anti-histone H1 antibody (Fig. 2).

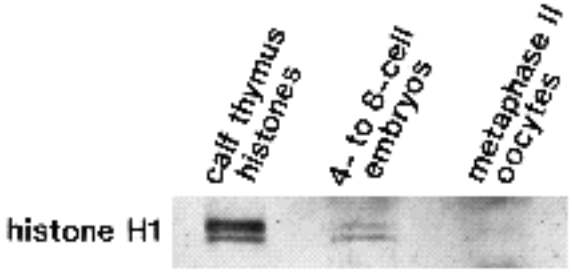


Fig. 2. Immunoblot analysis of histone H1 in oocytes and embryos. Lysates were prepared from 2820 metaphase II oocytes and 1480 late 4-cell or early 8-cell embryos, subjected to electrophoresis in polyacrylamide gels, transferred to nitrocellulose paper, and reacted with the anti-histone H1 antibody. Calf thymus histones were loaded on the same gel to identify the position of histone H1.

Fig. 3. Immunofluorescent staining of cleavage-stage embryos. For each embryo, the left panel shows the location of the nuclei as revealed by Hoechst 33258 fluorescence and the right panel shows the distribution of histone H1 as revealed by Texas Red fluorescence. (A) 2-cell embryo (48 hours post-hCG). No nuclear histone H1 is detectable. (B) 4-cell embryo (56 hours post-hCG). No nuclear histone H1 is detectable. (C) 4-cell embryo (68 hours post-hCG). Histone H1 is detectable in the nucleus of each cell, but not in the nucleus of the second polar body (arrow). (D) 8-cell embryo (68 hours post-hCG). Histone H1 is detectable in the nucleus of each cell, but not in the nucleus of the second polar body (arrow). (E) Histone H1 is present on mitotic chromosomes.

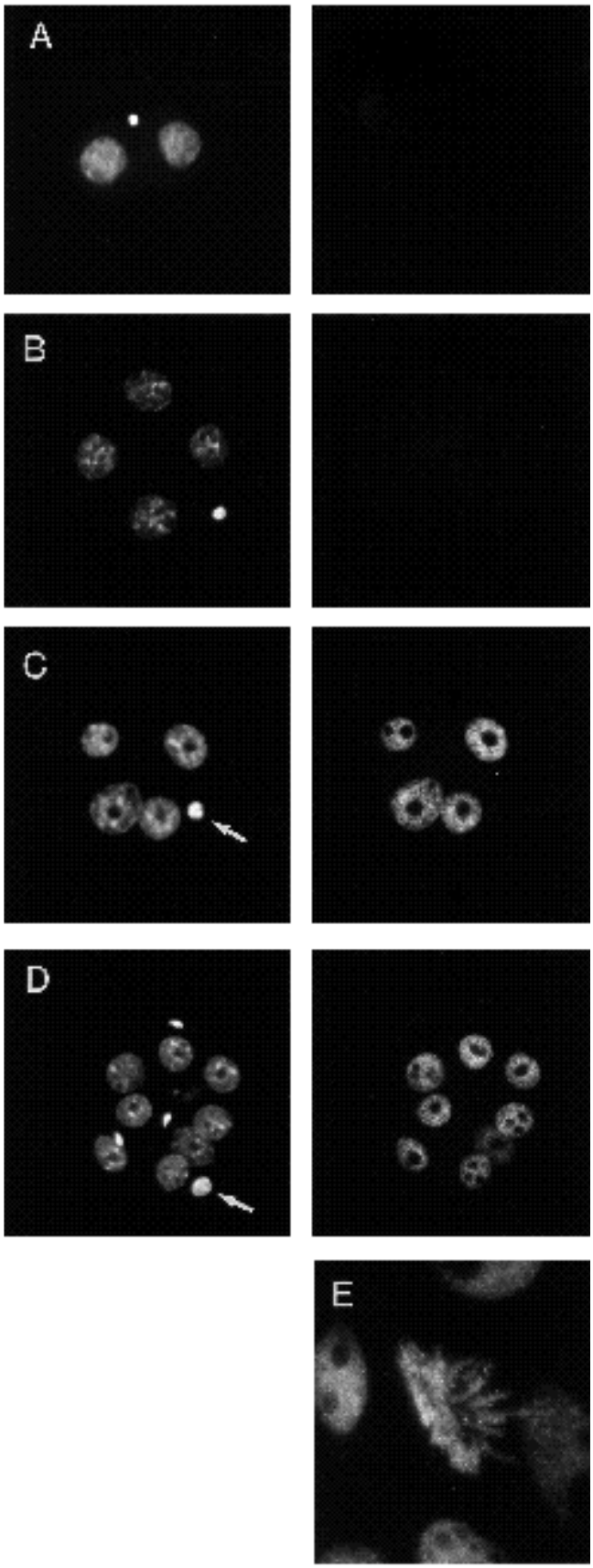
The 4- to 8-cell embryos contained two prominent immunoreactive species that co-migrated with calf thymus histone H1. In contrast, the oocytes did not contain detectable immunoreactive material at this position. Several bands of higher M_r not co-migrating with known histones were observed in both oocytes and 4- to 8-cell embryos. These results confirmed that oocytes did not contain a detectable quantity of somatic histone H1.

Appearance of somatic histone H1 at the 4-cell stage

To determine when somatic histone H1 first appeared during embryogenesis, embryos at different stages of preimplantation development were fixed and processed for immunofluorescence. The results of this experiment are illustrated in Fig. 3 and summarized in Table 1.

Embryos fixed at the 1-cell or 2-cell stage never displayed nuclear fluorescence. At the 4-cell stage, some embryos contained no fluorescent nuclei, as observed at the earlier stages (Fig. 3B). In other embryos, however, one or more of the nuclei were clearly fluorescent (Fig. 3C). The intensity of fluorescence varied among nuclei, occasionally even within a single embryo. The nucleus of the second polar body, which could be identified by its small size, was never fluorescent. At metaphase, the condensed chromosomes were fluorescent (Fig. 3E), which confirmed that the antibody detected chromosome-associated histone H1. Thus, histone H1 was detected in a proportion of nuclei in 4-cell embryos.

To analyze the basis of the variable histone H1 immunoreactivity in 4-cell embryos, these embryos were examined at two time points. In our experiments, embryos were at the 2-cell stage at 48 hours post-hCG. Most of these reached the 4-cell stage by 54-56 hours post-hCG and were classified as early 4-cell embryos. By 66-68 hours post-hCG, some embryos contained between 5 and 8 cells, indicating that they had begun third cleavage, whereas the others still contained 4 cells. This latter group were classified as late 4-cell embryos. When early 4-cell embryos were reacted with the anti-histone H1 antibody, the large majority contained no fluorescent nuclei (Table 1). A small fraction contained one or more fluorescent nuclei. In contrast, almost all of the late 4-cell embryos contained fluorescent nuclei (Table 1). Furthermore, in most cases, all four nuclei of these embryos were



fluorescent. These observations indicated that somatic histone H1 became detectable in cell nuclei during the third embryonic cell cycle.

To determine whether histone H1 remained immunologically detectable during subsequent preimplantation development, embryos were examined at the 5- to 8-cell, morula and blastocyst stages. All embryos examined at these stages contained fluorescent nuclei (Fig. 3D; Table 1). At the 5- to 8-cell stage, each nucleus was fluorescent in almost every embryo, although rarely non-fluorescent nuclei were observed. Among the morulae and blastocysts, each nucleus was fluorescent in all cases. Thus, once somatic histone H1 appeared at the 4-cell stage, it remained present in all cells of the preimplantation embryo.

Role of transcription

To analyze the regulation of the appearance of somatic histone H1 during the 4-cell stage, embryos were cultured in the presence of the transcriptional inhibitor, α-amanitin. The dose employed has previously been shown to inhibit essentially all transcription in mouse embryos (Kidder et al., 1985). In the first experimental series, α-amanitin was added to 2-cell embryos at 48 hours post-hCG. The embryos were cultured until 68-74 hours post-hCG, when untreated 4-cell embryos contain somatic histone H1 (Table 1). Most of the embryos incubated in the presence of the drug cleaved to form 4-cell embryos (Table 2). As transcription during the 2-cell stage is required for second cleavage (Flach et al., 1982), this observation confirms that the embryos were at the late 2-cell stage when the drug was added. When these embryos were fixed and reacted with the antibody, the large majority contained no fluorescent nuclei (Table 2). This result indicated that transcriptional activity beyond the late 2-cell stage was required for the appearance of somatic histone H1 in nuclei.

In the second experimental series, embryos were transferred to medium containing α-amanitin at 54-56 hours post-hCG, incubated until 68-74 hours post-hCG, fixed and processed as above. Because of the natural developmental asynchrony within a population of embryos, these embryos could be subdivided into three groups. These three groups showed different patterns of histone H1 immunoreactivity. The first group consisted of embryos that were at the 2-cell stage when the drug was added and cleaved to the 4-cell stage during subsequent incubation. Almost none of these embryos contained fluorescent nuclei when fixed at 68-74 hours post-hCG (Fig. 4A, Table 2), confirming the dependence of histone H1 immunoreactivity on transcription beyond the late 2-cell stage.

The second group consisted of embryos that were at the 4-cell stage when the drug was added and remained at this stage during incubation. About half of these embryos contained fluorescent nuclei when fixed (Fig. 4B, Table 2). In these cases, however, the intensity of fluorescence was weak, and often had a punctate appearance rather than being uniformly distributed through the nuclei. The remaining embryos in this group did not contain any fluorescent nuclei. This result suggested that the appearance of a normal quantity of somatic histone H1 required transcription during the 4-cell stage.

The third group consisted of the embryos that were at the

4-cell stage when the drug was added and progressed to the 5- to 8-cell stage during subsequent incubation. In this group, fluorescent nuclei were observed in every embryo (Table 2). As observed in the second group, this fluorescence in many cases was weak and often displayed a punctate pattern. In some embryos, however, the intensity of nuclear fluorescence was indistinguishable from that of embryos not exposed to the drug. Thus, embryos that had completed the transcription required for third cleavage had also completed the transcription required to produce somatic histone H1.

Role of translation

To test whether the appearance of somatic histone H1 required translation of mRNA, embryos were transferred to medium containing the protein synthesis inhibitor, puromycin, and incubated until 68 hours post-hCG. 2-cell embryos transferred to medium containing puromycin at 48 hours post-hCG failed to cleave to the 4-cell stage (Table 3). When reacted with the anti-histone H1 antibody, none of these embryos contained fluorescent nuclei (Table 3). 4-cell embryos transferred to puromycin at 54-56 hours post-hCG failed to undergo third cleavage. About one-third of these embryos showed weak nuclear fluorescence, whereas the remaining two-thirds contained no fluorescent nuclei. These results confirmed that protein synthesis during the 4-cell stage was required for the appearance of somatic histone H1.

Role of DNA replication

The observation that somatic histone H1 was absent at the early 4-cell stage yet present at the late 4-cell stage suggested that its appearance might be linked to progression through the cell cycle. In most proliferating cells, histone synthesis occurs primarily during S-phase, although a low level of synthesis continues throughout the cell cycle (Wu and Bonner, 1981; Wu et al., 1984), and histone synthesis declines rapidly when DNA replication is inhibited (Wu and Bonner, 1981). A similar link between DNA replication and histone synthesis has been described in rabbit preimplantation embryos (Matheson and Schultz, 1980). To test whether the appearance of somatic histone H1 at the 4-cell stage in mice was linked to DNA replication, embryos at the late 2-cell stage (48 hours post-hCG) were transferred to medium containing the DNA polymerase inhibitor, aphidicolin. Preliminary experiments established that 100 μg/ml aphidicolin, but not 10 μg/ml aphidicolin, prevented detectable DNA synthesis in 4-cell embryos as monitored by immunofluorescent detection of the thymidine analogue, BrdU (not shown). Embryos were incubated at the higher drug concentration until 68-70 hours post-hCG, fixed and processed for immunofluorescence.

The 2-cell embryos incubated in the presence of aphidicolin cleaved to form 4-cell embryos, indicating that they had completed S-phase of the second cell cycle when the drug treatment began. When these 4-cell embryos were reacted with the anti-histone H1 antibody, a weak nuclear fluorescence (Fig. 4C) was observed in 20 of 25 embryos. In 18 of these 20, all four nuclei displayed this weak fluorescence. The remaining 5 embryos did not contain fluorescent nuclei. These results indicated that, although embryos cultured in the presence of aphidicolin could produce somatic histone H1, the amount was quantitatively reduced compared to untreated embryos.

Discussion

Absence of somatic histone H1 in the nuclei of oocytes and early embryos

We used an antibody directed against somatic histone H1 to examine the distribution of histone H1 in oocytes and preimplantation embryos of the mouse. This antibody detected histone H1 in the nuclei of embryonic blastomeres and of cumulus granulosa cells, indicating that it recognizes histone H1 species present in proliferating and terminally differentiated cells of the mouse. However, no somatic histone H1 could be detected in the nuclei of immature oocytes or embryos up to the early 4-cell stage.

Several observations make it unlikely that some feature of these cells or their chromatin structure rendered histone H1 inaccessible to the antibody. First, histone H1 microinjected into immature oocytes accumulated in the nucleus, where it

Table 2. *The development of histone H1 immunoreactivity in embryos cultured in the presence of α-amanitin*

Time added (hours post-hCG)	Embryo stage	Time fixed (hours post-hCG)	Embryo stage	Number of embryos	+*	−
48	2-cell	68-74	4-cell	61	5	56
54-56	2-cell	68-74	4-cell	14	2	12
54-56	4-cell	68-74	4-cell	22	10	12
54-56	4-cell	68-74	5- to 8-cell	18	18	0

*Embryos containing one or more fluorescent nuclei were scored as positive.

Table 3. *The development of histone H1 immunoreactivity in embryos cultured in the presence of puromycin*

Time added (hours post-hCG)	Embryo stage	Time fixed (hours post-hCG)	Embryo stage	Number of embryos	+*	−
48	2-cell	68	2-cell	35	0	35
56	4-cell	68	4-cell	33	8	25

*Embryos containing one or more fluorescent nuclei were scored as positive.

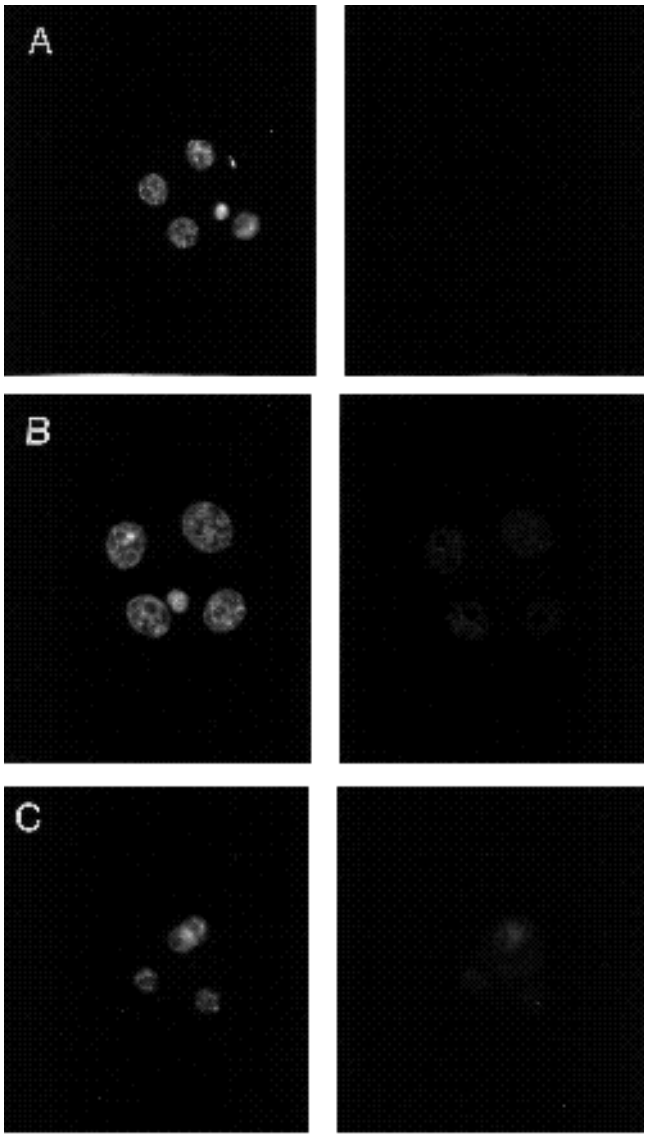


Fig. 4. Immunofluorescent staining of embryos exposed to inhibitors of RNA and DNA synthesis. For each embryo, the left panel shows the location of the nuclei as revealed by Hoechst 33258 fluorescence and the right panel shows the distribution of histone H1 as revealed by Texas Red fluorescence. (A) Embryo exposed to α-amanitin beginning at 54 hours post-hCG and fixed at 68 hours post-hCG. No histone H1 is detectable. (B) Embryo exposed to α-amanitin beginning at 54 hours post-hCG and fixed at 68 hours post-hCG. Histone H1 is detectable in each blastomere nucleus, but the quantity is reduced compared to embryos not exposed to the drug (Fig. 3C). (C) Embryo exposed to aphidicolin begining at 48 hours post-hCG and fixed at 68 hours post-hCG. A small quantity of histone H1 is detectable in each nucleus. The slight difference in apparent nuclear diameter among the panels is due to the fact that the oocytes become flattened to different extents when they are mounted onto slides.

was immunologically detectable. Second, oocyte lysates analyzed by immunoblotting did not contain detectable histone H1, although at least two histone H1 subtypes were present in lysates of 8-cell embryos. Third, the fact that histone H1 was not detected in the early cleavage-stage embryos implies that, if a specific feature of chromatin structure prevented immunological detection of histone H1, it must be shared by oocyte and early embryonic nuclei including those of transcriptionally active 2- and early 4-cell embryos. These considerations strongly suggest that the nuclei of oocytes and embryos up to the early 4-cell stage contain little or none of the histone H1 subtypes that are present in somatic cells.

This interpretation is consistent with results previously observed in other organisms. In general, oocytes and early embryos lack the histone H1 subtypes found in other cells, but instead contain a unique histone H1 subtype. These oocyte subtypes have a larger M_r and, where their amino acid content has been analyzed, contain less lysine than the somatic cell subtypes (Poccia, 1986; Smith et al., 1988; Ohsumi and Katagiri, 1991). Furthermore, in sea urchins (Pehrson and Cohen, 1984) and frogs (Wolffe, 1989; Smith et al., 1988), the histone H1 subtypes of oocytes appear to be immunologically distinct from the somatic subtypes. In the case of the mouse, meiotically immature oocytes synthesize a lysine-containing protein that accumulates in the nucleus and has a M_r slightly higher than histone H1 (Wassarman et al., 1979). These results together with those reported here suggest that, as in non-mammalian species, mouse oocytes could contain a histone H1 subtype that is immunologically distinct from the somatic subtypes.

Appearance of somatic histone H1 during embryogenesis

Histone H1 became detectable in embryonic nuclei beginning at the 4-cell stage. Among the embryos examined shortly after cleavage from the 2-cell stage, a small proportion contained histone H1 in one or more nuclei. But when the embryos were incubated for an additional 12 hours before examination, almost all of them contained histone H1. In most of these late 4-cell embryos, histone H1 was present in all four nuclei. This result indicates that development of histone H1 immunoreactivity at the 4-cell stage was associated with progression through the cell cycle. Among embryos examined at subsequent stages of development, histone H1 was present in all cell nuclei. As it is known that the cell cycles become progressively less synchronous during development (Gamow and Prescott, 1970), this observation indicates that histone H1 was detectable at all stages of the cell cycle in these older embryos. Thus, once somatic histone H1 appeared at the 4-cell stage, it remained detectable throughout subsequent development. It may be concluded that somatic histone H1 becomes associated with chromatin at the 4-cell stage and remains present through subsequent cell divisions.

The appearance of somatic histone H1 at the 4-cell stage was prevented by exposing embryos to the RNA polymerase II inhibitor, α-amanitin, suggesting that it requires de novo transcription. When the drug was added to the culture medium at the late 2-cell stage, almost none of the embryos produced detectable histone H1 and, when it was added at the early 4-cell stage, about one-third of the embryos did not produce detectable histone H1. These results imply that this transcription occurs during the third cell cycle. It may also be noted that, among embryos exposed to α-amanitin beginning at the early 4-cell stage, about two-thirds produced detectable histone H1 whereas only one-third reached the 5-

to 8-cell stage. This result may suggest that the transcription required to produce histone H1 occurs in advance of that required for the third cleavage.

We also observed that the quantity of somatic histone H1 in the nuclei of 4-cell embryos was substantially reduced when the third round of DNA replication was inhibited. Thus, there was a link between the development of histone H1 immunoreactivity and DNA replication. This link may reflect the manner in which histone gene expression is regulated. Many histone genes, known as replication-dependent variants, encode subtypes that are synthesized primarily during S-phase (review: van Holde, 1989). By contrast, replacement-type variants encode subtypes whose synthesis is not restricted to S-phase (Wu and Bonner, 1981; Sittman et al., 1983). When DNA replication is inhibited, histone synthesis declines rapidly, due to reduced transcription and degradation of the mRNA encoding the replication-dependent subtypes (Sittman et al., 1983; Graves et al., 1987). Based on this data, our results can be explained by postulating that, when the third round of embryonic DNA replication is prevented by addition of aphidicolin to culture medium, synthesis of somatic histone H1 is correspondingly inhibited.

Somatic histone H1 and the switch from maternal to embryonic control of development

The observations that somatic histone H1 first appears after the activation of embryonic transcription, which occurs at the 2-cell stage in mice (reviewed by Telford et al., 1990), and that its appearance is sensitive to α-amanitin, suggest that this histone H1 is synthesized from embryo-encoded transcripts. In the following scheme, it is proposed how somatic histone H1 synthesis could be developmentally regulated during early mouse embryogenesis. According to this scheme, oocytes do not contain somatic histone H1, although they may contain a distinct subtype. At the 2-cell stage, embryos begin transcriptional activity. Because transcription of the replication-dependent genes is linked to S-phase, however, histone mRNA is not synthesized at this time. After the second cleavage division occurs and the 4-cell embryos begin DNA replication, active transcription of the histone genes begins, and somatic histone H1 is synthesized and becomes associated with chromatin. The precise timing of appearance of somatic histone H1 in 4-cell embryos is thus postulated to occur as a result of the combined effects of the timing of embryonic transcriptional activation and the cellular regulation of histone synthesis. This proposal could be tested by examining when somatic histone H1 becomes detectable in mammals in which the embryonic genome becomes active at later cleavage stages.

The notion that the appearance of somatic histone H1 in 4-cell embryos reflects a switch from maternal to embryonic control is consistent with current knowledge of the patterns of histone synthesis in mouse oocytes and embryos. The histone pool in mouse oocytes is estimated to be about 50 pg (Wassarman and Mrozak, 1981; Schultz, 1986). The level of histone synthesis in 1-cell embryos is low compared to later stages (Kaye and Wales, 1981; Kaye and Church, 1983), and the amount of mRNA encoding core histones declines substantially by the 2-cell stage (Giebelhaus et al., 1983; Graves et al., 1985), suggesting that the histone pool existing in oocytes is not significantly supplemented by post-fertilization histone synthesis from maternal mRNA. Should this be the case, then the oocyte pool must supply the histone required during DNA replication until embryo-encoded histones are produced. As the histone content of diploid mouse nuclei is 6 pg, a 50 pg pool would be sufficient to produce the four tetraploid nuclei of a G_2-stage 4-cell embryo. Thus, the maternally supplied histone pool may become exhausted at approximately the same stage that somatic histone H1, which we speculate to be embryo-encoded, is first detected.

The experiments described here have revealed that somatic histone H1 becomes associated with chromatin at the 4-cell stage in mouse embryos. Changes in the histone complement of chromatin during early mouse development may not be restricted to histone H1. A comparison of the mRNA populations that encode core histones between oocytes and blastocysts indicates that the relative abundance of different transcripts differs in the two cell types (Graves et al., 1985), which suggests that the core histone subtypes present on embryonic chromatin may change during this time. The consequences of such developmentally regulated changes in histone composition remain to be elucidated. It is tempting to speculate, however, that they could underly the changes in nuclear potential revealed by nuclear transplantation studies, or generate or propagate the differential imprinting of the maternally and paternally derived genomes.

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