INTRODUCTION

Following fertilization, early development is directed by maternally inherited mRNAs and proteins. The zygotic genome becomes transcriptionally active at a time that is species-dependent (Telford et al., 1990), after which the embryo synthesizes new transcripts that are required for further development. In the mouse, several lines of evidence indicate that zygotic gene activation (ZGA) definitely occurs by the 2-cell stage (see Schultz, 1993 and references therein), and more recent evidence suggests that transcription is evident as early as G2 of the first cell cycle (Latham et al., 1992; Ram and Schultz, 1993; Matsumoto et al., 1994; Christians et al., 1995). The molecular basis for the maternal to zygotic transition in the mouse, however, is poorly understood.

Changes in chromatin structure, rather than activation of the transcription machinery itself, may underlie the molecular basis for ZGA and continued gene expression in the preimplantation embryo. For example, functional RNA polymerase II (Latham et al., 1992) and transcription factors are present in the 1- and 2-cell embryos (e.g., Vernet et al., 1992; Wiekowski et al., 1993; Ram and Schultz, 1993). Moreover, although enhancers are required for efficient reporter gene expression in the 2-cell embryo, this requirement is relieved by sodium butyrate treatment (Wiekowski et al., 1991, 1993), probably as a result of increased histone acetylation and consequent changes in chromatin structure (Candido et al., 1978).

A growing body of evidence suggests that chromatin structure plays an integral role in the regulation of transcription (Wolffe, 1994 and references therein). Chromatin structure is inherently transcriptionally repressive, since the nucleosomal subunit structure can prevent transcription factors from binding to their cognate sequences in DNA (Wolffe, 1991, 1994). Conversely, in other situations a positioned nucleosome is essential for transcriptional initiation (Wolffe, 1994). In at least some cases, the role of chromatin can be modulated by post-translational modification of core histones.

Histones are susceptible to a wide spectrum of modifications that include phosphorylation, ADP-ribosylation, ubiquitination, and acetylation (Wolffe and Dimitrov, 1993; Turner, 1991, 1993). Acetylation in particular is receiving much attention, since hyperacetylation is highly correlated with the potential for transcriptional activity (e.g., Jeppesen and Turner, 1993; Braunstein et al., 1993; Lin et al., 1989). Increased histone acetylation can facilitate the binding of some transcription factors to nucleosomes in vitro (Lee et al., 1993).

To explore the role of changes in histone acetylation in ZGA in the mouse, we initiated studies using laser-scanning confocal microscopy and a panel of antibodies specific for different acetylated forms of histone H4 (Turner and Fellows, 1994). Using immunofluorescent labeling and laser-scanning confocal microscopy, we show that isoforms of histone H4 acetylated on lysine 5, 8 and/or 12 (H4.Ac5-12), as well as RNA polymerase II, become enriched at the nuclear periphery around the time of zygotic gene activation, i.e., the 2-cell stage, in the preimplantation mouse embryo. In contrast, DNA and H4 acetylated on lysine 16 are uniformly distributed throughout the cytoplasm. Culture of embryos with inhibitors of histone deacetylase trichostatin A and trapoxin results in an increase in the (1) amount of acetylated histone H4 detected by immunoblotting, (2) intensity and sharpness of the peripheral staining for H4.Ac5-12, and (3) relative rate of synthesis of proteins that are markers for zygotic gene activation. The enhanced staining for H4.Ac5-12 at the nuclear periphery seems to require DNA replication, but appears independent of cytokinesis or transcription, since its development is inhibited by aphidicolin but not by either cytochalasin D or α-amanitin. Lastly, the restricted localization of H4.Ac 5-12 is not observed in the 4-cell embryo or at later stages of preimplantation development. These results suggest that changes in chromatin structure underlie, at least in part, zygotic gene activation in the mouse.

Key words: mouse embryo, gene expression, chromatin, histone H4, histone acetylation
MATERIALS AND METHODS

Embryo collection and culture
One-cell embryos were collected from superovulated CF-1 female mice (Harlan) mated to B6D2/J males (Jackson Laboratory) and synchronized as previously described (Ram and Schultz, 1993). Embryo culture was conducted in CZB medium (Chatot et al., 1989) at 37°C in an humidified atmosphere containing 5% CO2 in air. Two- and 4-cell embryos were flushed from the oviducts 44 hours and 61 hours post-hCG, respectively.

Immunofluorescence and laser-scanning confocal microscopy
Laser-scanning confocal microscopy of fixed embryos was performed as previously described (Worrad et al., 1994). An RNase step was included when the DNA was stained with YOYO-1 (Molecular Probes Inc.), since this dye binds to both DNA and RNA. Following embryo permeabilization, the embryos were washed in 2 drops of PBS/PVP, placed in blocking solution (0.1% BSA, 0.01% Tween-20, PBS) for 15 minutes, and the protocol resumed as previously described (Turner and Fellows, 1989; Worrad et al., 1994). Antibody R20/12 was used at a 1:400 dilution and detected with 5.0 

Metabolic radiolabeling of 2-cell embryos and SDS-PAGE
Unsynchronized 1-cell embryos in S phase were cultured overnight in KSOM (Erbach et al., 1994) containing 2-amanitin (24 

RESULTS

Increased localization of histone H4 acetylated on lysines 5, 8 and/or 12 at the nuclear periphery in 2-cell mouse embryos
Changes in chromatin structure, rather than changes in the intrinsic activity of the transcription apparatus, may be criti-
cally involved in ZGA and continued gene expression. The finding that treatment with the histone deacetylase inhibitor sodium butyrate relieves a requirement for enhancers for efficient expression of reporter genes (Wiekowski et al., 1991, 1993) suggests that histone acetylation may play a role in bringing these changes about. To test this we have used immunolabeling with antibodies to different forms of acetylated histone H4 and laser-scanning confocal microscopy to search for changes in the temporal and spatial localization of isoforms of acetylated histone H4 during early preimplantation development.

As anticipated, DNA was essentially uniformly distributed throughout the nucleoplasm in 1- and 2-cell embryos (Fig. 1), although there was an increase in staining intensity around the nucleolus-like-bodies. Likewise, a uniform nuclear staining pattern was observed following staining with antibody R14/16 in both 1- and 2-cell embryos. In contrast, an increased staining intensity was observed at the nuclear periphery when antibodies R12/8, R20/12, or R40/5 were used (Fig. 1). The increased staining at the nuclear periphery was clearly evident in the 2-cell embryos and could be detected in S/G2 of the 1-cell embryo. In the latter case, the female pronucleus preferentially, i.e., more frequently, exhibited this type of peripheral staining pattern (Figs 2 and 3); the female pronucleus is the smaller of the two pronuclei.

It was unlikely that the uniform staining pattern observed with antibodies R12/8, R20/12 and R40/5 was due to restricted access of the antibodies to the interior of the nucleus, since antibody R14/16 resulted in a uniform staining pattern and was clearly able to penetrate the nucleus. The uniform staining pattern observed with R14/16 also minimized the possibility (without entirely excluding it) that the peripheral staining with antibodies R12/8, R20/12 or R40/5 was due to their inability to bind to the appropriate modified lysine residue in chromatin in the nuclear interior, since residues 5, 8, 12 and 16 all lie in close proximity along the N-terminal domain of H4.

Immunostaining with an antibody to RNA polymerase II showed that, at least by the 2-cell stage, this enzyme also displayed an increased localization at the nuclear periphery, similar to that observed for histone H4.Ac5, H4.Ac8, and H4.Ac12. The antibody recognizes the carboxy-terminal domain (CTD) of the large subunit, a region that is subject to post-translational phosphorylation and is therefore likely to be relatively accessible (e.g., Lu et al., 1992; Serizawa et al., 1994). Staining for RNA polymerase II was also observed in the nuclear interior. Attempts to co-localize directly H4.Ac5-16 and RNA polymerase II by double immunolabeling have, as yet, been unsuccessful in that, for reasons that remain unclear, initial labelling with either of these antibodies prevents subsequent labelling with the other (data not shown).

The frequency of the peripheral staining for histone H4, detected with antibodies R12/8, R20/12, R40/5, and RNA polymerase II in 1- and 2-cell embryos is shown in Fig. 2. The frequency of staining at the nuclear periphery for acetylated isoforms of histone H4 increased during the first cell cycle and was higher in the female pronucleus during all time points examined. In contrast, RNA polymerase II was not localized at the nuclear periphery during the first cell cycle, but was localized to a significant extent in the 2-cell embryos and this incidence increased somewhat with cell cycle progression (53% in early G2 and 74% in mid-G2).

Chromatin assembled on newly replicated DNA contains diacetylated histone H4, in which lysines 12 and 5 are selectively acetylated (Sobel et al., 1995). Thus, it was possible that the enrichment of H4.Ac5 and H4.Ac12 at the nuclear periphery was due to diacetylated histone H4 assembled on newly replicated DNA. This possibility was tested by pulse labeling 1-, 2-, or 4-cell embryos with BrdU and locating the sites of DNA replication by immunolabeling with anti-BrdU antibody and laser-scanning confocal microscopy. After a 15 minute pulse (the shortest for which we could detect a signal), labeling revealed punctate nuclear fluorescence throughout the nucleus with no evidence of enhanced staining at the nuclear periphery (Fig. 3). In the same experiment the embryos were also stained with antibody R20/12 and the staining patterns observed were similar to those shown in Fig. 1 (data not shown). It should also be noted that although no incorporation of BrdU was observed 12 hours post-PN formation (data not shown), the enhanced staining at the nuclear periphery using antibody R20/12 or R40/5 was present (Fig. 1).

**Lack of effect of culture on the development of nuclear peripheral staining pattern for H4.Ac12 and RNA polymerase II in 2-cell embryos**

Oxidative stress during culture can detrimentally affect development and frequently manifests itself during the 2-cell stage (Johnson and Nasr-Esfahani, 1994). Addition of antioxidants or superoxide dismutase, however, can overcome this inhibitory effect (Johnson and Nasr-Esfahani, 1994; Vernet et al., 1993).

It was unlikely that the increased staining of these acetylated isoforms of histone H4 at the nuclear periphery in the 2-cell embryos was a culture artifact. First, the addition of superoxide dismutase (6,000 units/ml) to the culture medium did not alter the incidence of this staining pattern in 1-cell embryos cultured to the 2-cell stage when the embryos were stained with R20/12; in each instance 100% of the 2-cell embryos displayed the enhanced staining pattern at the nuclear periphery when the cells were examined in early G2. Also, addition of superoxide dismutase had no discernible effect on the intensity of staining (data not shown). Second, when embryos were stained with antibody R20/12, the enhanced staining pattern at the nuclear periphery was observed for 2-cell embryos flushed from the oviduct (Fig. 4) and the incidence and intensity of this pattern was similar to that for embryos that developed from the 1-cell to 2-cell stage in vitro, i.e., about 90% and 70% for embryos that developed in vitro or in vivo, respectively. Moreover, a similar fraction of the 2-cell embryos that developed in the presence or absence of superoxide dismutase, 69% and 67%, respectively, revealed an enhanced peripheral staining for RNA polymerase II at the nuclear periphery.

**Nuclear peripheral staining of H4.Ac5 and H4.Ac12 is restricted to the 2-cell stage**

To ascertain if the nuclear peripheral staining pattern for H4.Ac5 and H4.Ac12 observed in 2-cell embryos persisted at later stages of development in the context of continuing activity of the zygotic genome, flushed 2-cell and 4-cell embryos were analyzed (Fig. 4). Whereas 72% of the flushed 2-cell embryos had peripheral nuclear staining of H4.Ac12, none of the flushed 4-cell embryos examined exhibited this staining pattern (Fig. 4B); similar results were observed when antibody R40/5 was
Fig. 1. Confocal immunofluorescent microscopy images of synchronized 1-cell and 2-cell embryos stained for DNA, the different isoforms of acetylated histone H4, or RNA polymerase II. (A-E) YOYO-1 staining modified for DNA, (F-J) antibody R14/16, (K-O) antibody R12/8, (P-T) antibody R20/12, (U-Y) antibody R40/5, and (Z-DD) anti-RNA polymerase II antibody. All individual antibody panels are arranged as follows: one-cell 6 hours after PN formation (S phase); 1-cell 9 hours after PN formation (mid-late S phase); 1-cell 12 hours after PN formation (G2/M); 2-cell 44 hours post-hCG (G2); and 2-cell 47 hours post-hCG (mid G2). The number of 1-cell embryos analyzed at 6 h, 9 h, and 12 hours after PN formation and the number of 2-cell embryos analyzed at 44 hours and 47 hours post-hCG was: 23, 24, 23, 24, 24 for YOYO; 8, 10, 10, 10, 10 for antibody R14/16; 31, 30, 30, 28, 21 for antibody R12/8; 73, 74, 68, 76, 71 for antibody R20/12; 10, 10, 10, 10, 9 for antibody R40/5; and 31, 31, 28, 76, 78 for antibody to RNA polymerase II, respectively. For each treatment group the experiment was performed at least 2 times, except for R14/16 and R40/5, for which a single experiment was conducted. Shown are representative examples.
used (data not shown). Thus, this spatially restricted staining pattern was transient; it correlated with the onset of ZGA but then dissipated by the 4-cell stage. In addition, the uniform staining pattern was observed in 8-cell embryos, and in the cells of the inner cell mass and trophectoderm at the blastocyst stage when antibody R20/12 was used (data not shown).

**Effect of inhibiting cytokinesis, transcription, or DNA replication on the nuclear peripheral staining pattern for H4.Ac5 and H4.Ac12**

ZGA is independent of cytokinesis, since it occurs in cytochalasin-treated, cleavage-arrested 1-cell embryos that are chronologically at the 2-cell stage (Petzdolt, 1984; Poueymirou and Schultz, 1987). Preferential localization of H4.Ac12 at the nuclear periphery continued to occur in cytochalasin-treated embryos (Fig. 5). Similar results were observed when antibody R40/5 was used (data not shown).

The highest incidence of this staining pattern was observed in 2-cell embryos in G2, which corresponds to the major burst of transcription; ZGA is characterized by two putative periods of transcription: an initial period that occurs at G1 (~1-1.5

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**Fig. 2.** Frequency of enhanced staining at the nuclear periphery during the first and second cell cycle. (A) Antibody R12/8, (B) antibody R20/12, (C) antibody R40/5, (D) anti-RNA polymerase II antibody. For antibody R12/8, the number of 1-cell embryos analyzed at 6 h, 9 h, and 12 hours after PN formation was 31, 30, 30, respectively, and the number of 2-cell embryos analyzed at 44 hours and 47 hours post-hCG was 28 and 21, respectively. For antibody R20/12, the number of 1-cell embryos analyzed at 6 h, 9 h, and 12 hours after PN formation was 73, 74, and 68, respectively, and the number of 2-cell embryos analyzed at 44 hours and 47 hours post-hCG was 76 and 71, respectively. For antibody R40/5, the number of 1-cell embryos analyzed at 6 h, 9 h, and 12 hours after PN formation was 10 at each time point, and the number of 2-cell embryos analyzed at 44 hours and 47 hours post-hCG was 10 and 9, respectively. For RNA polymerase II, the number of 1-cell embryos analyzed at 6 h, 9 h, and 12 hours after pronuclei formation was 31, 31, and 28, respectively, and the number of 2-cell embryos analyzed at 44 hours and 47 hours post-hCG was 78 and 63, respectively. Solid bars, staining in the male PN; open bars, staining in the female PN; stippled bars, staining in cultured 2-cell embryos. For each group the experiment was performed at least 2 times. Shown are representative examples.

**Fig. 3.** Confocal immunofluorescent microscopy images of 1-, 2-, and 4-cell embryos labeled with BrdU. The embryos were incubated in medium containing BrdU for 15 minutes prior to fixation and processing for laser-scanning confocal microscopy. (A) 1-cell embryo 6 hours post-PN formation, (B) 2-cell embryo, (C) 4-cell embryo. The experiment was performed two times and similar results were obtained. The total number of embryos analyzed was 16, 15, and 17, for 1-, 2-, and 4-cell embryos, respectively. All of the embryos revealed a similar staining pattern.
hours after cleavage to the 2-cell stage) and a second one that occurs at 44-52 hours post-hCG (Bolton et al., 1984). Transcription was not required, however, for the preferential localization of H4.Ac12 to the nuclear periphery, since it was observed in 1-cell embryos cultured to the 2-cell stage in the presence of α-amanitin (24 μg/ml) (Fig. 5); such treated embryos will cleave to the 2-cell stage, but not to the 4-cell stage (Poueymirou and Schultz, 1989). Again, α-amanitin also had no effect on the staining pattern when antibody R40/5 was used (data not shown).

ZGA occurs in aphidicolin-treated cleavage-arrested 1-cell embryos that are chronologically at the 2-cell stage (Howlett, 1986; Poueymirou and Schultz, 1987). We treated 1-cell embryos in S phase with aphidicolin, allowed them to develop until they were chronologically at the 2-cell stage and then examined the location of H4.Ac5-12. It was found that such treatment substantially suppressed the formation of this staining pattern with both antibodies R20/12 (Fig. 5), and R40/5 and R12/8 (data not shown).

**Effect of histone deacetylase inhibitors, trichostatin A and trapoxin, on gene expression and staining pattern of H4.Ac12**

The steady-state level of histone acetylation in a cell is established by the dynamic balance of histone acetyltransferases and deacetylases, and deacetylase inhibitors can therefore be used in experiments to correlate increased levels of histone acetylation with changes in cell function. Sodium butyrate, the most frequently used inhibitor of histone deacetylases, has been used in many studies, but has many nonspecific effects (Kruh, 1982). We used instead two relatively new, potent and specific inhibitors of histone deacetylases, trichostatin A and trapoxin, to determine how treatment of embryos with these compounds affected the nuclear peripheral localization of acetylated histone H4. Trichostatin A is a reversible inhibitor (Yoshida et al., 1990), whereas trapoxin is an irreversible inhibitor (Kijima et al., 1993); each works in the nM range.

One-cell embryos cultured in the presence of these inhibitors cleaved to the 2-cell stage and these 2-cell embryos contained a marked increase in the amount of histone H4 acetylated on lysine 12 as detected by immunoblotting (Fig. 6). The amount of histone H4 acetylated on lysine 12 in trichostatin A-treated and trapoxin-treated embryos was 3.5 and 4.5 times higher, respectively, than in untreated embryos. Thus, the acetate groups on histone H4 in the early mouse embryo are clearly turning over. It should be noted that in Xenopus laevis embryos, in which the maternal histone H4 pool is diacetylated and becomes deacetylated during development, sodium butyrate treatment has very little effect on increasing the amount of hyperacetylated histone H4 up to the midblastula transition, which is when ZGA occurs (Dimitrov et al., 1993).

Correlated with this increase in histone H4 acetylation was an increase in the relative rate of synthesis of a family of structurally related proteins of Mr = 70,000 (Conover et al., 1991) (Fig. 7). [The synthesis of these proteins is inhibited by α-amanitin and restricted to the 2-cell stage. We refer to them as the transcription-requiring complex (TRC; Conover et al., 1991); they are accepted markers for ZGA.] The stimulatory effect on the relative rate of TRC synthesis evoked by trapoxin treatment was greater than the response to trichostatin A. Nevertheless, the ratio of the stimulatory effect on TRC synthesis to the fold-increase in acetylation of histone H4 was essentially similar, i.e., 0.49 for trichostatin A and 0.51 for trapoxin. It should be noted that butyrate treatment also increases the relative rate of synthesis of the TRC, as well stimulating the expression of reporter genes in mouse embryos (Wiekowski et al., 1993).

To ascertain the morphological effects of trichostatin A and trapoxin, 1-cell embryos were cultured from S to G2/M phase of the first cell cycle or S of the first cell cycle to G2 phase of the second cell cycle in the presence of these inhibitors and fluorescence was analyzed by laser-scanning confocal microscopy (Fig. 8). In the 1-cell embryo, neither trichostatin A nor trapoxin had a significant effect on the localization of either DNA (Fig. 8A-C) or H4.Ac16 (Fig. 8D-F), although the intensity of the immunofluorescent staining appeared enhanced. In contrast, staining with antibody R20/12 revealed an increase in the intensity of the fluorescence located at the nuclear periphery and the rim appeared tighter (Fig. 8G-I). As with the effect of these inhibitors on the increase in histone acetylation and gene expression, the effect of trapoxin appeared greater than that of trichostatin A. Moreover, the effect of these inhibitors was more pronounced for the female pronucleus. Similar results were obtained for 2-cell embryos following incubation with these deacetylase inhibitors (Fig. 8J-R), i.e., an increase in staining intensity at the nuclear periphery with an associated tightening of the rim of fluorescence, and trapoxin having the more pronounced effect. Similar results were obtained when the treated embryos were stained with R40/5 and R12/8 (data not shown).

**DISCUSSION**

We report here that an enrichment of histone H4 acetylated at lysines 5, 8, and/or 12 (H4.Ac5-12) and RNA polymerase II at the nuclear periphery is evident in the 2-cell mouse embryo.

![Fig. 4. Confocal immunofluorescent microscopy images of flushed 2-cell and 4-cell mouse embryos stained with antibody R20/12. (A) Flushed 2-cell embryo (44 hours post-hCG). (B) Flushed 4-cell embryo (61 hours post-hCG). The percentage of flushed 2-cell embryos that exhibited nuclear rimming was 72% compared with 0% of the flushed 4-cell embryos. The number of flushed 2-cell embryos analyzed was 78 and the number of flushed 4-cell embryos was 30.](image-url)
Gene expression in preimplantation mouse embryo

This enrichment is transient and not observed in the 4-cell embryo or at later stages of preimplantation development. It therefore coincides with the maternal to zygotic transition. The formation of this localized distribution of H4.Ac5-12 is independent of cytokinesis and transcription, but requires DNA synthesis. Treatment of the embryos with inhibitors of histone deacetylases increases both the amount of acetylated histone H4 detected by immunoblotting, showing that turnover of histone H4 acetates occurs during these very early developmental stages, and the intensity and sharpness of the fluorescent signal attributable to acetylated histone H4 at the nuclear periphery. These inhibitors also increased the relative rate of synthesis of proteins that are markers for ZGA.

In interpreting the immunostaining results, it is important to realize that staining with any one antibody does not, in itself, indicate whether or not lysine residues other than the one for which the antibody is specific are also acetylated. Thus, immunostaining with R40/5 shows the presence of histone H4 acetylated at lysine 5 but gives no indication as to whether or not the isoforms detected are also acetylated at other sites. In adult somatic cells lysine 5 is usually acetylated only in the tri- and tetra-acetylated isoforms, while lysine 16 is the predominant acetylated residue in mono-acetylated histone H4 (Turner et al., 1989; Thorne et al., 1990). If this is also the case in the early mouse embryo, then the nuclear peripheral staining with R40/5 would indicate the presence of hyperacetylated histone H4 at the periphery. The very small amount of material available from these early developmental stages has so far prevented the successful completion of experiments to address this point, but it is worth noting that recent experiments with histones from cultured mouse embryonic stem cells show a reproducible shift in the pattern of acetylation of histone H4 lysines compared to adult cells (J. Lavender, A. Keohane, and B.M. Turner, unpublished observations). Thus, while histone H4 at the nuclear periphery may well be hyperacetylated, mono- or di-acetylated isoforms may also be present. The situation of the nuclear interior is clearer. As chromatin in the nuclear interior labeled with R14/16, but not as strongly with the other antisera, it seems that the predominant acetylated histone H4 isoform in this part of the nucleus is mono-acety-

Fig. 5. Confocal immunofluorescent microscopy images of synchronized mouse 2-cell embryos (44 hours post-hCG) treated with different inhibitors and stained with R14/16 or R20/12. α-amanitin (24 µg/ml), cytochalasin D (1 µg/ml) or aphidicolin (3 µg/ml) were added to 1-cell embryos 6 hours after PN formation and they were then cultured to the 2-cell stage (44 hours post-hCG) at which time they were fixed and processed for laser-scanning confocal microscopy. (A-D) Embryos stained with antibody R14/16; (E-H) embryos stained with antibody R20/12. (A,E) Control embryos; (B,F) embryos cultured in the presence of α-amanitin; (C,G) embryos cultured in the presence of cytochalasin D,; (D,H) embryos cultured in the presence of aphidicolin. The number of 2-cell embryos analyzed for the control, α-amanitin, cytochalasin D and aphidicolin samples stained with antibody R14/16 was 27, 24, 18, and 25, respectively. The number of 2-cell embryos analyzed for the control, α-amanitin, cytochalasin D and aphidicolin samples stained with antibody R20/12 was 25, 26, 22, and 24, respectively. The experiment was performed two times and similar results were obtained in each case. Shown are representative examples.

Fig. 6. Immunoblot of acetylated histone H4 recognized by antibody R20/12 in 2-cell embryos cultured in the presence of either trichostatin A or trapoxin. Unsynchronized 1-cell embryos were placed in CZB and cultured overnight (lane 1) or placed in CZB containing either trichostatin A (lane 2) or trapoxin (lane 3) and cultured overnight. Extracts were prepared from 250 embryos and immunoblot analysis was then conducted as described under Materials and Methods. The experiment was performed three times and similar results obtained in each case; shown is a representative example.
lated at lysine 16. Whatever the precise molecular explanation for the staining patterns we observe, the results show quite clearly that H4 isoforms acetylated at different lysine residues have different, preferred intranuclear locations at the earliest stages of development.

We considered the possibility that the enhanced staining for H4.Ac5 and H4.Ac12 at the nuclear periphery reflects the incorporation of histone H4 diacetylated on lysines 12 and 5 into newly replicated chromatin (Sobel et al., 1995). Three observations argue against this possibility. First, we could find no evidence by pulse labeling with BrdU that DNA replication is preferentially restricted to the nuclear periphery in 1-, 2-, and 4-cell embryos. It should be noted, however, that the shortest pulse we were able to use was 15 minutes and so the rapid transfer of newly replicated chromatin from the periphery to the nuclear interior (with concomitant H4 deacetylation) would disguise peripheral synthesis. Second, virtually no sign of increased fluorescence at the periphery in either the male or female pronucleus is observed with antibodies to H4.Ac5 6 hours post-PN formation (Figs 1U, 2C) at which time the cells are synthesizing DNA and presumably assembling it into chromatin. In contrast, peripheral staining is observed at this time when antibody R20/12 is used. If the enhanced fluorescence at the nuclear periphery represents newly incorporated diacetylated histone H4 modified at lysines 12 and 5, one would anticipate that staining with antibody R20/12 or R40/5 would each yield enhanced peripheral staining, and such is not the case. Third, 12 hours post-PN formation the embryos display the enhanced staining at the nuclear periphery but at this time the embryos are in G2 and not synthesizing DNA, which was confirmed by the absence of BrdU incorporation (data not shown).

Although the results listed above argue against a direct link between ongoing chromatin assembly and the peripheral location of acetylated histone H4, other results clearly establish that the distribution is dependent on continued DNA synthesis. When 1-cell embryos in S phase are cultured in the presence of the DNA synthesis inhibitor aphidicolin until they are chronologically at the 2-cell state, a marked reduction is observed in the localization of acetylated H4 at the nuclear periphery. Although ZGA, as assessed by the synthesis of the TRC, occurs in these embryos at essentially the same time as in control embryos, examination of the fluorograms suggests that the relative rate of synthesis of the TRC is reduced when compared to that in control embryos (Howlett, 1986; Poueymirou and Schultz, 1987); it should also be noted that in these studies aphidicolin was added when the 1-cell embryos were already in S phase. Therefore, DNA replication may be important for ZGA. Inspection of the photomicrographs showing the distribution of acetylated H4 in aphidicolin-treated embryos (Fig. 5) – the aphidicolin was added during S phase in these experiments – suggests that there is in fact a residual enrichment of H4.Ac5-12 at the nuclear periphery, albeit less than in the controls and with a more diffuse staining pattern. Thus, it is still possible that the effect of aphidicolin on the location of acetylated H4 may be through aphidicolin’s suppressive effects on transcription rather than on DNA replication per se. It is of course quite possible, perhaps even likely, that DNA replication and transcription are closely coupled processes in the early embryo. Such a coupling has obvious attractions as a mechanism for establishing patterns of gene expression for subsequent developmental stages. The dissociation of nucleosomes and chromatin remodeling that accompany DNA replication provides a window of opportunity in which transcription factors can compete with histones for DNA, bind to their DNA sequences and thereby recruit the assembly of a functional transcription complex (Laybourn and Kadonaga, 1991; Felsenfeld, 1992; Wolffe, 1991, 1994).

The increased concentration and co-localization of H4.Ac5-12 and RNA polymerase II at the nuclear periphery in the 2-cell embryo may indicate that this is potentially a region of high transcriptional activity. Although this was not examined directly in this study, autoradiography of sectioned 8-cell bovine embryos that were metabolically labeled with [3H]uridine reveals blastomeres with an intense ring of grains

Fig. 7. Relative rate of TRC synthesis in unsynchronized 2-cell embryos treated with α-amanitin, trichostatin A, or trapoxin. (A) One-dimensional gel electrophoresis of 1-cell embryos cultured to the 2-cell stage in the presence of α-amanitin, trichostatin A, or trapoxin and metabolically labeled with [35S]methionine from 52-54 hours post-hCG (late G2); equal number of counts (35,000 cpm) were loaded per lane. Lane 1, untreated 2-cell embryos; lane 2, α-amanitin-treated embryos; lane 3, trichostatin A-treated embryos; lane 4, trapoxin-treated embryos. The brackets ( ) denote TRC and the arrows (→) denote a protein of Mr~100x103 whose synthesis is also inhibited by α-amanitin. Asterisks (∗) denote a family of proteins of Mr~30x103-35x103 that undergo cell cycle-dependent protein phosphorylation (Howlett, 1986). The experiment was performed three times and similar results were obtained in each case. Shown is a representative example. (B) Relative rate of TRC synthesis. The relative rate of TRC synthesis was quantified as described under Materials and Methods. In these experiments, the signal in the region of the TRC for embryos cultured in the presence of α-amanitin was subtracted from that for the TRC to control for the synthesis of polypeptides in this region of the gel whose synthesis is not affected by α-amanitin. The results are expressed as the mean ± s.e.m. The differences between the relative rate of synthesis for the TRC in the drug-treated samples relative to that in the control is statistically significant (P<0.5, paired t-test).
located at the nuclear periphery (Pavlok et al., 1993). A body of evidence suggests that ZGA occurs in the late 4-cell to early 8-cell embryo in cattle (Telford et al., 1990; Barnes and First, 1991). The absence of an increased peripheral localization of RNA polymerase II in the 1-cell embryos may reflect that although these embryos are transcriptionally active, the level

Fig. 8. Confocal immunofluorescent microscopy images of synchronized mouse 1-cell and 2-cell embryos treated with trapoxin or trichostatin A. (A-I) 1-Cell embryos; (J-R) 2-cell embryos; (A,D,G,J,M,P) control embryos; (B,E,H,K,N,Q) trapoxin-treated embryos; (C,F,I,L,O,R) trichostatin A-treated embryos. (A-C) 1-cell embryos at G2/M phase stained with YOYO-1; (D-F) 1-cell embryos at G2/M phase stained with antibody R14/16; (G-I) 1-cell embryos at G2/M phase stained with antibody R20/12; (J-L) 2-cell embryos 44 hours post-hCG stained with YOYO-1; (M-O) 2-cell embryos 44 hours post-hCG stained with antibody R14/16; (P-R) 2-cell embryos 44 hours post-hCG stained with antibody R20/12. For YOYO-1 staining, the number of 1-cell embryos analyzed for control, trapoxin-, and trichostatin A-treated embryos was 20, 20, and 20, and for 2-cell embryos 20, 17, and 20, respectively. For antibody R14/16, the number of 1-cell embryos analyzed for control, trapoxin-, and trichostatin A-treated embryos was 20, 20, and 20 and for 2-cell embryos 29, 29, and 29, respectively. For antibody R20/12, the number of 1-cell embryos analyzed for control, trapoxin-, and trichostatin A-treated embryos was 26, 20, and 20 and for 2-cell embryos 45, 29, and 29, respectively. The experiment was performed two times and similar results were obtained in each case. Shown are representative examples.
is low when compared to the 2-cell embryo (Ram and Schultz, 1993; Matsumoto et al., 1994; Christians et al., 1995).

Also of interest is the difference in the frequency of the enhanced staining pattern for acetylated histone H4 at the pronuclear periphery between the male and female pronuclei; a higher frequency is observed for the female pronucleus. Although the molecular basis for this is not known, there are several examples of such differences. These include differences in the ability of the male and female pronuclei to support expression of reporter genes (Ram and Schultz, 1993; Wiekowski et al., 1993); requirement for enhancers for efficient expression of the reporter genes (Martines-Salas et al., 1989; Wiekowski et al., 1991, 1993; Majumder et al., 1993) and the effectiveness of butyrate treatment in relieving this requirement (Wiekowski et al., 1993); pronuclear concentration of transcription factors (Worrad et al., 1994); extent of chromosome condensation following fusion with a metaphase II-arrested egg (Cierny and Czołowska, 1993); and as mentioned above, differences in the response to trichostatin A and trapoxin with respect to fluorescence intensity following staining with antibody R20/12.

Increased histone acetylation has been correlated with the potential for transcription, though not with transcription itself. Some or all of histones in chromatin around the chicken β-globin gene are acetylated in cells where the gene is likely to be expressed, even at developmental stages at which the gene is not transcribed (Hebbes et al., 1988, 1992, 1994). H4 on the mating type genes in yeast is not acetylated in cells where these genes are to be silenced, but is acetylated in cells where they are to be transcribed (Braunstein et al., 1993). Importantly, histone H4 acetylation occurs under conditions where transcription is appropriate, even in mutants where the genes cannot actually be transcribed (Braunstein et al., 1993). Also, in metaphase chromosomes, histone H4 acetylation is increased in regions known to be enriched for coding DNA, even though transcription is minimal at this stage of the cell cycle (Jeppesen and Turner, 1993). Very recently, immunoprecipitation of chromatin fragments from human HL-60 cells with antibodies to acetylated histone H4 used in the experiments reported here has confirmed that histone H4 associated with coding DNA is acetylated while histone H4 associated with heterochromatin is not, but has also shown that the level of histone H4 acetylation within or adjacent to coding regions does not change with shifts in transcriptional activity (O’Neill and B.M. Turner, 1995). Collectively, these results suggest that histone H4 acetylation is involved in chromatin remodelling during differentiation and development, part of its function being to protect selected regions of the genome from long-term transcriptional silencing. Its role, if any, in the short-term switching of gene activity remains less clear. The acetylation of histones other than H4 is likely to have different functional effects.

Thus, the development of the enhanced localization of H4.Ac5-12 at the nuclear periphery may be an essential step in preparing for ZGA. Selective acetylation of histone H4 may provide a chromatin groundstate that is transcriptionally permissive, i.e., it is necessary but not sufficient for transcription. Transcription will occur if the appropriate complement of transcription factors is present and active, and can gain access to their DNA-binding sequences. We would suggest that as transcription initiates in the mouse embryo, it may do so preferentially at the nuclear periphery that bears chromatin enriched in acetylated histone H4. The development of this regionalized domain of chromatin that is transcriptionally permissive is under maternal control and forms regardless of whether transcription can or cannot take place, since it occurs in 2-cell embryos that are cultured from the 1-cell stage in the presence of β-amanitin. The spectrum of genes that are then expressed reflects the complement and concentration of maternally inherited active transcription factors and the array of promoter and enhancer elements present on genes that exist within this nuclear domain. The formation of chromatin bearing nucleosomes containing these acetylated isoforms of histone H4 may be required to counter the formation of a transcriptionally repressive state that develops following the first mitosis (Wiekowski et al., 1991, 1993; Henery et al., 1995) and is thus involved in sustaining continued gene expression that is necessary for further development.

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