Histone macroH2A1 is concentrated in the inactive X chromosome of female preimplantation mouse embryos

Carl Costanzi[1], Paula Stein[2], Diane M. Worrad[2,*], Richard M. Schultz[2] and John R. Pehrson[1,‡]

[1]Department of Animal Biology, School of Veterinary Medicine and [2]Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA
*Present address: Department of Pathology, Fox Chase Cancer Center, Philadelphia, PA 19111, USA
‡Author for correspondence (e-mail: pehrson@vet.upenn.edu)

SUMMARY

MacroH2As are core histone proteins with a hybrid structure consisting of a domain that closely resembles a full-length histone H2A followed by a large nonhistone domain. We recently showed that one of the macroH2A subtypes, macroH2A1.2, is concentrated in the inactive X chromosome in adult female mammals. Here we examine the timing of the association of macroH2A1.2 with the inactive X chromosome during preimplantation mouse development in order to assess the possibility that macroH2A1 participates in the initiation of X inactivation. The association of macroH2A1.2 with one of the X chromosomes was observed in 50% of blastocysts, occurring mostly, if not exclusively, in extraembryonic cells as was expected from previous studies, which indicated that X inactivation in embryonic lineages happens after implantation. Examination of earlier embryonic stages indicates that the association of macroH2A1 with the inactive X chromosome begins between the 8- and 16-cell stages. Of the changes that are known to happen during X inactivation in preimplantation embryos, the accumulation of macroH2A1 appears to be the earliest marker of the inactive X chromosome and is the only change that has been shown to occur during the period when transcriptional silencing is initiated.

Key words: Preimplantation mouse embryo, X chromosome inactivation, Chromatin, Histone

INTRODUCTION

X inactivation involves several changes in the structure or properties of the inactive X chromosome that are believed to play a role in the initiation and/or maintenance of transcriptional silencing (Heard et al., 1997). These include hypermethylation of CpG islands, hypoacetylation of core histones, asynchronous replication, and the accumulation of a large noncoding nuclear RNA from a gene called *Xist*. *Xist* RNA coats the inactive X chromosome in cis and is generally thought to be directly involved in the inactivation process, though how it mediates transcriptional silencing is unknown.

We recently showed that the inactive X chromosome in adult female mammals is also characterized by a high concentration of the core histone macroH2A1.2, and can be distinguished in interphase nuclei as a large macroH2A-dense domain called a macrochromatin body (MCB) (Costanzi and Pehrson, 1998). MacroH2A1.2 is one of the three known macroH2A subtypes. Two subtypes, macroH2A1.1 and 1.2, are produced by alternate splicing of the *macroH2A1* gene (Pehrson et al., 1997; Rasmussen et al., 1999). The third subtype, macroH2A2.2, is produced from a separate gene (C. Costanzi and J. R. Pehrson, unpublished results). All three subtypes have a similar structure, consisting of an amino-terminal domain that closely

resembles a full-length histone H2A followed by a large nonhistone domain that comprises 57% of the protein (Fig. 1A). The H2A domain appears to replace conventional H2A in a subset of nucleosomes and it was estimated that one in 30 nucleosomes in rat liver would contain macroH2A, assuming one macroH2A per nucleosome (Pehrson and Fried, 1992). The majority of the nonhistone region appears to have evolved from a gene of unknown function that originated prior to the appearance of eukaryotes (Pehrson and Fuji, 1998). On the basis of the homology of the nonhistone region to proteins involved in the replication of viral RNA, it was hypothesized that the nonhistone region may bind RNA and that the localization of macroH2A1 to the inactive X chromosome might be mediated by an interaction between the nonhistone region and *Xist* RNA (Pehrson and Fuji, 1998). A connection between *Xist* and macroH2A1 has been established in a mouse fibroblast model, in which deletion of part of the *Xist* locus from the inactive X chromosome leads to the loss of macroH2A1 association (Csankovszki et al., 1999).

Two experimental systems have been used to examine the early events of X inactivation. One system uses female mouse embryonic stem (XX-ES) cells or embryonal carcinoma cells in vitro (Martin et al., 1978; Rastan and Robertson, 1985). In culture XX-ES cells undergo random X chromosome

inactivation about 2 days after they are induced to differentiate (Keohane et al., 1996). X-linked genes are silenced shortly after the accumulation of stable *Xist* RNA and the transition to late replication, but 2 days before H4 deacetylation (Keohane et al., 1996). In this system the association of macroH2A1 with the inactive X is a late event, occurring about 5 days after inactivation (Mermoud et al., 1999). Methylation of CpG islands appears to be a late event, occurring weeks after initiation of differentiation (Keohane et al., 1996).

The early events of X inactivation have also been examined in vivo using harvested mouse preimplantation embryos. In this system X chromosome inactivation occurs between the 8-cell stage, where both chromosomes appear to be active, and the blastocyst stage, where X inactivation has occurred in most cells (Adler et al., 1977; Epstein et al., 1978; Kratzer and Gartler, 1978; Monk and Kathuria, 1977; Singer-Sam et al., 1992). X inactivation in blastocysts is imprinted, i.e. the paternal X chromosome is preferentially inactivated (Takagi and Sasaki, 1975; Takagi et al., 1978), and is thought to involve only extraembryonic cells, because X inactivation in embryonic tissues appears to be random (Lyon, 1961) and to occur after implantation (Gardner et al., 1985).

It is not clear what changes in the paternal X chromosome are responsible for initiation of imprinted X inactivation. Knockout experiments showed that *Xist* is required for initiation of X inactivation (Marahrens et al., 1997), but accumulation of *Xist* RNA appears to occur significantly before inactivation. For instance, in early mouse development accumulated *Xist* RNA from the paternal X chromosome is present as early as the 4-cell stage (Kay et al., 1994) and a large domain of accumulated *Xist* RNA was detected in the nuclei of all cells of 8-cell embryos (Sheardown et al., 1997). These results indicate that the accumulation of *Xist* RNA is a prerequisite for initiation of X inactivation, but that other factors are required. Asynchronous replication of the inactive X chromosome (Sugawara et al., 1985) and inactive X-specific methylation of CpG islands (Grant et al., 1992; Lock et al., 1987) do not appear to be involved in initiation of X inactivation in preimplantation embryos because both of these changes seem to occur after transcriptional silencing.

In the present study we examine the timing of the accumulation of macroH2A1 in the inactive X chromosome in mouse preimplantation embryos, in order to assess whether macroH2A1 accumulation could participate in the initiation of imprinted X inactivation. Also, by ordering macroH2A1 accumulation relative to other known changes in the inactive X chromosome in this system, we can assess whether accumulation of macroH2A1 depends on these other changes.

MATERIALS AND METHODS

Western blot

50 μl of nuclear isolation buffer (0.4 M mannitol, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 0.5 mM EGTA, 15 mM triethanolamine, pH 7.4, containing 0.2% Triton X-100, 0.3 mM phenylmethanesulfonylfluoride and 6 μg/ml aprotinin) were added to approximately 10 μl of minimal essential medium (MEM) containing 150-300 blastocysts. The suspension was incubated for 10 minutes on ice with occasional agitation. The sample was centrifuged at 15,000 *g* for 5 minutes and the pellet resuspended

in 50 μl of nuclear isolation buffer, incubated for 5 minutes on ice and centrifuged. The pellet was dissolved in SDS-sample buffer (Laemmli, 1970) containing 20 mM Tris(2-carboxyethyl)-phosphine (Pierce) as a reductant, and briefly heated in a boiling water bath. The proteins were separated by SDS-gel electrophoresis alongside a similar amount of rat liver nuclear extract. The gel loading was based on the estimated DNA content of the samples. The separated proteins were transferred onto a polyvinylidene difluoride membrane, and the membrane was blocked as previously described (Pehrson et al., 1997). The blocked membrane was incubated overnight at 4°C with antibodies against the nonhistone region of rat macroH2A1.2 (Costanzi and Pehrson, 1998), washed, and then incubated with peroxidase-conjugated donkey anti-rabbit IgG. The signal was detected using SuperSignal West Femto Maximum Sensitive Substrate (Pierce).

Immunofluorescence

Embryo collection and indirect immunofluorescence of mouse preimplantation embryos were performed as previously described (Worrad et al., 1994). 8-cell embryos were harvested between 67.75 and 68.75 hours post human chorionic gonadotropin injection; morulae between 72 and 90 hours; early blastocysts between 90 and 91 hours; and blastocysts at 96 hours.
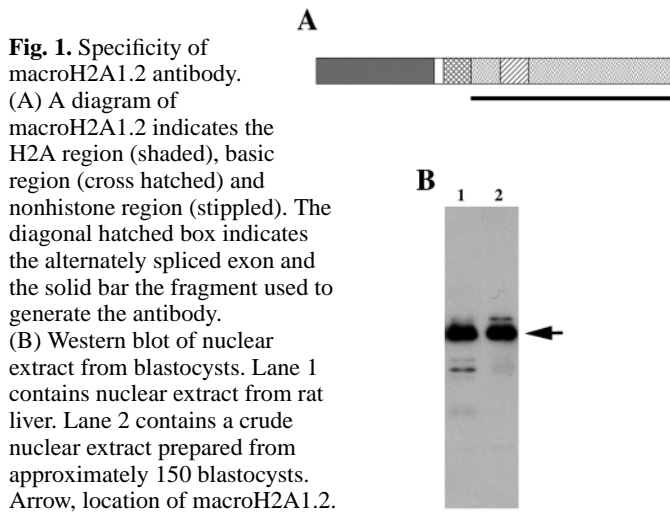
Immunofluorescence/fluorescence in situ hybridization

Blastocysts in 20 μl of MEM were added to a cytospin cuvette. 170 μl of acidic tyrode solution (−polyvinylpyrrolidone) were added directly to the cuvette and incubated at room temperature for 2 minutes to remove the zona pellucida. The acid was neutralized with 10 μl of 10× PBS and the embryos spun onto gelatin-coated slides at 225 *g* for 10 minutes. The embryos were fixed in 4% formaldehyde for 5 minutes and then the reaction was quenched in 100 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% Triton X-100. The slides were then blocked with 5% nonfat dry milk in TBSN (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.06% NP40) for 1 hour. Embryos were incubated overnight at 4°C with the primary antibody diluted in TBSN/milk. The next day the slides were washed with TBSN/milk, incubated with the Texas Red-labeled mouse anti-rabbit IgG (Jackson Immunoresearch), washed with TBSN/milk, and then with PBS. Embryos were again fixed in 4% formaldehyde then quenched in 100 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% Triton X-100 for 20 minutes. The embryos were then rinsed in TBS, fixed in 3:1 methanol:acetic acid for 20 minutes, washed in 2× SSC (0.3 M NaCl, 30 mM sodium citrate), incubated with 100 μg/ml RNase A in 2× SSC for 30 minutes at 37°C, washed again in 2× SSC, dehydrated through a 70%-85%-100% ethanol series and air dried. Hybridization was performed using biotinylated mouse X chromosome paint probe (Oncor), according to the manufacturer's instructions. For hybrid detection a layer of FITC-labeled streptavidin (Jackson Immunoresearch) was followed by a layer of biotin-labeled anti-streptavidin (Accurate Chemical) and another layer of FITC-streptavidin, as previously described (Costanzi and Pehrson, 1998). Embryos were counterstained with Hoechst 33258, mounted and examined on a Leica TCS-SP laser-scanning confocal microscope.

RESULTS

Antibody specificity

For immunofluorescence we used an affinity-purified antibody directed against the nonhistone region of rat macroH2A1.2 (Costanzi and Pehrson, 1998) (Fig. 1A). We assessed the specificity of this antibody by western blot analysis of nuclear extracts prepared from blastocysts. This revealed a band of the appropriate mobility (Fig. 1B). The specificity of macroH2A1.2 staining was further assessed in a control

Fig. 1. Specificity of
macroH2A1.2 antibody.
(A) A diagram of
macroH2A1.2 indicates the
H2A region (shaded), basic
region (cross hatched) and
nonhistone region (stippled). The
diagonal hatched box indicates
the alternately spliced exon and
the solid bar the fragment used to
generate the antibody.
(B) Western blot of nuclear
extract from blastocysts. Lane 1
contains nuclear extract from rat
liver. Lane 2 contains a crude
nuclear extract prepared from
approximately 150 blastocysts.
Arrow, location of macroH2A1.2.

Table 1. Occurrence of X-macrochromatin bodies (MCB) in mouse preimplantation embryos

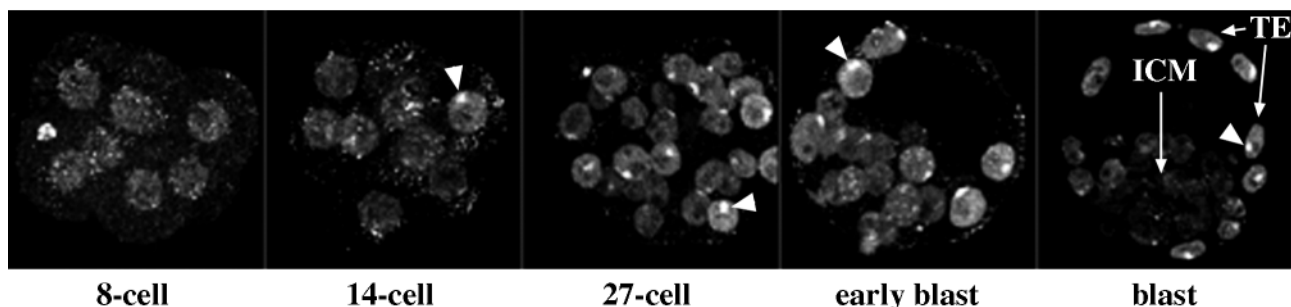| Embryo stage	X-MCB-containing embryos/total (%)
8-cell	0/24 (0)
Morula (9-32 cells)	18/45 (40)
Blastocyst (>32 cells)	40/81 (49)

embryos showed diffuse or speckled macroH2A1.2 staining, and many nuclei also contained several smaller MCBs. These MCBs exhibited no cell-type specificity and varied in number from 0 to 20 per nucleus (see below).

To determine whether the large MCBs were female-specific and corresponded to one of the two X chromosomes, we performed X chromosome 'paint' fluorescence in situ hybridization (FISH) together with macroH2A1.2 immunofluorescence. The DNA was counterstained with Hoechst 33258. All of the blastocysts that contained large MCBs had two X chromosomes, and thus were female. Virtually all of the large MCBs colocalized with one of the two X chromosomes (Fig. 3), with a distinct region of faint Hoechst staining. This distinct X-MCB DNA domain seen with Hoechst in embryos is similar to the distinct X-MCB DNA domain seen with propidium iodide in adult tissues (Costanzi and Pehrson, 1998). In contrast, the nonX-MCBs were present in both male and female embryos, and were associated with Hoechst-dense DNA domains (Fig. 4). These Hoechst-dense regions contained centric heterochromatin, as determined by FISH using a γ-satellite (Vissel and Choo, 1989) probe (Fig. 4D). It was not clear whether these non-X MCBs included satellite DNA or were only adjacent to centromeric heterochromatin.

Metaphase chromosomes also stained for macroH2A1.2 and in many cases we were able to discern a single brightly stained chromosome (Fig. 5).

Timing of MCB appearance in preimplantation embryos

To determine the timing of MCB appearance in preimplantation development, we examined 8-cell embryos, morulae and early blastocysts by immunofluorescence. We detected macroH2A1.2 protein in the nuclei of all cells at all

experiment where the primary antibody was initially incubated with a tenfold molar excess of the glutathione-S-transferase-macroH2A1.2 nonhistone region fusion protein used to generate the antibody. In this control experiment all immunofluorescent staining of blastocysts was eliminated (not shown).

MacroH2A1 is concentrated in the inactive X chromosome in blastocysts

To examine the possibility that macroH2A1 associates with the inactive X chromosome in preimplantation embryos we used immunofluorescence confocal microscopy to look for MCBs in blastocysts. Approximately half of the blastocysts examined had cells that contained a single large MCB (Table 1). The presence of such MCBs in half of the embryos is consistent with these MCBs corresponding to the inactive X, which is only present in female embryos. In those blastocysts that contained MCBs, we detected a single large MCB in virtually all trophectoderm cells (trophectoderm cells form the extraembryonic outer epithelial layer of the blastocyst, marked TE in Fig. 2, and are the predominant cell type at this stage). Most cells in the inner cell mass (ICM) did not contain such an MCB, though a small number of MCB-containing cells (0-3 per embryo) were present in the ICM at this stage (the ICM will give rise to the embryonic lineages). Cell nuclei of all the


Fig. 2. Onset of macrochromatin body (MCB) appearance in mouse preimplantation embryos. Embryos were stained with antibody to macroH2A1.2 and viewed by fluorescence confocal microscopy (see Table 1). The 8-cell, 14-cell and 27-cell embryos are shown as a complete stack of confocal slices. The blastocysts (blast) are shown as a stack of central confocal slices to distinguish the outer trophectoderm layer from the inner cell mass. In some trophectoderm nuclei, the MCB is present in focal planes that are not shown. Arrowheads point to MCBs. Arrows indicate the locations of the inner cell mass (ICM) and trophectoderm (TE).
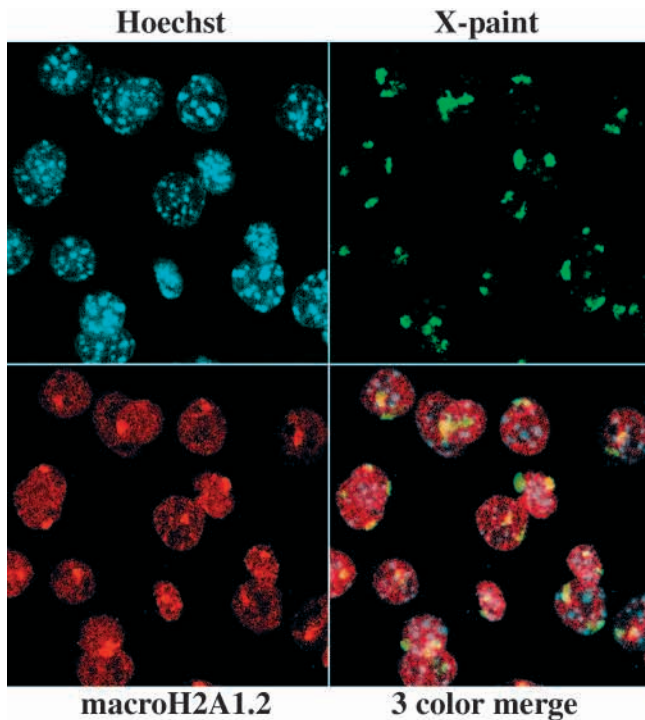
Fig. 3. Colocalization of MCBs with one of the X chromosomes. Blastocysts were stained for macroH2A1.2 (red), hybridized with an X chromosome paint probe (green), and stained with the DNA dye Hoechst 33258 (blue). A region of the trophectoderm of a blastocyst is shown. Colocalized MCBs and X chromosomes appear orange in the 3-color merged image.

developmental stages examined, though the intensity of the immunofluorescence was relatively low at the 8-cell stage and increased through the blastocyst stage. We also observed speckled cytoplasmic staining in 8-cell embryos and morulae that was mostly absent in blastocysts. Whether this represents cytoplasmic stores of macroH2A1 or merely crossreacting cytoplasmic epitopes is unknown. We did not detect MCBs in 8-cell embryos, whereas 40% of the morulae contained cells with a single large distinct MCB (Table 1 and Fig. 2). We detected MCBs in morulae as early as the 12-cell stage. In experiments where we double-stained the embryos with macroH2A1.2 antibody and Hoechst 33258, these early MCBs were intranuclear and associated with a distinct region of faint Hoechst staining, as seen with the X-MCBs of the blastocysts (Fig. 6). In the early blastocysts that contained MCBs, the cells on the outside of the embryos almost always contained an MCB, while the interior region of the embryo was composed of a mixture of MCB-containing and MCB-negative cells. Small nonX-MCBs were also present at these earlier stages of development, but we were able to distinguish these from X-MCBs by their association with regions that stained brightly with Hoechst 33258. Attempts to stain morulae for both macroH2A1 and X chromosomes did not produce satisfactory results due to a variety of technical problems, including the lower intensity of macroH2A1 staining obtained at these stages.

We conclude that the large MCBs we see in the morulae are X-MCBs based on the following criteria: (1) they are present in about half of the embryos; (2) they are relatively large in
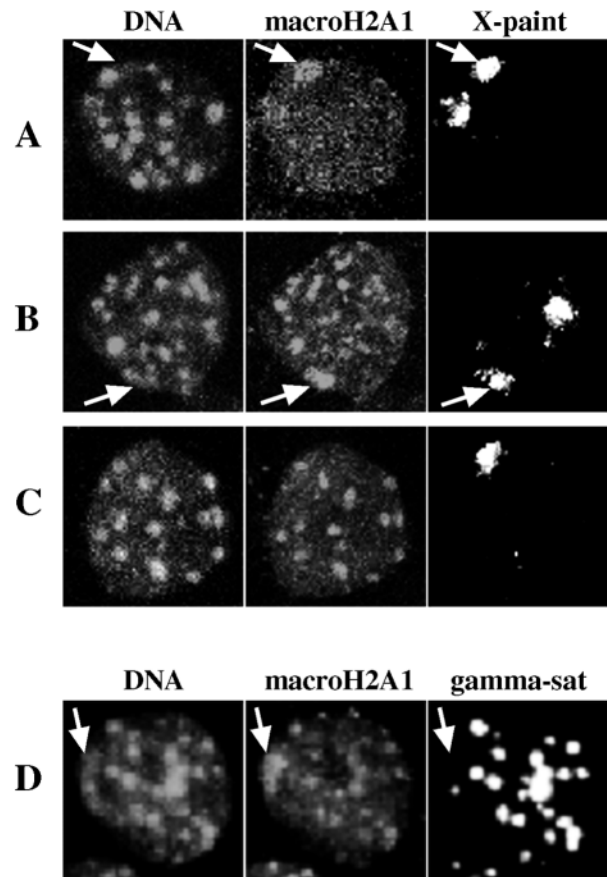

Fig. 4. X-MCBs and nonX-MCBs in blastocysts. Blastocysts were stained for macroH2A1.2 (macroH2A1), hybridized with an X chromosome paint probe (X-paint) or γ-satellite probe (gamma-sat), and stained with the DNA dye Hoechst 33258 (DNA). Stacked images of four trophoblast nuclei are shown from four different embryos. A, B and D are from female embryos and C is from a male embryo. X-MCBs are marked with arrows. The nucleus in example A does not contain nonX-MCBs, but B, C and D do.
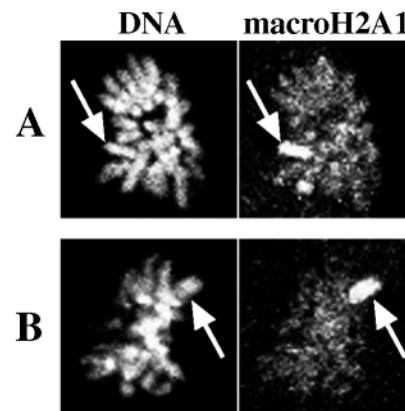
Fig. 5. Metaphase chromosomes in blastocysts. Shown are single optical sections through metaphase trophoblasts in two separate blastocysts stained for macroH2A1.2 and with the DNA dye Hoechst 33258. The individual chromosomes brightly stained with the macroH2A1.2 antibody are marked with arrows.
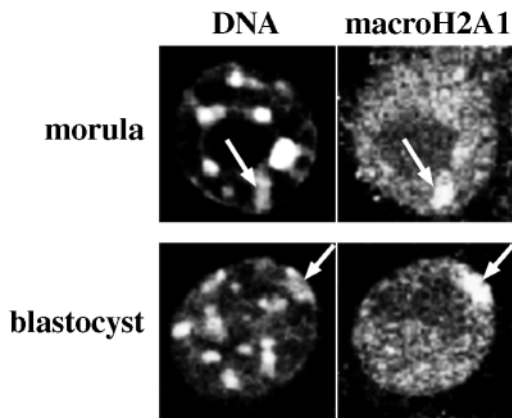
Fig. 6. X-MCBs in morula. Morulae and blastocysts were stained for macroH2A1.2 and with the DNA dye Hoechst 33258. Shown are single optical sections through an X-MCB containing a nucleus from each. X-MCBs are marked with arrows.

size; (3) they are localized to an X-MCB-like DNA domain, as determined by confocal microscopy. These criteria clearly distinguish the X-MCBs from the nonX-MCBs seen in embryos as well as the nonX-MCBs seen by conventional fluorescence microscopy in undifferentiated XX- and XY-ES cells (Mermoud et al., 1999), which have since been shown to be extranuclear structures (J. Mermoud, personal communication).

DISCUSSION

Assuming that the correspondence between female-specific X-MCBs and inactive X chromosomes demonstrated previously in adult tissues (Costanzi and Pehrson, 1998) also applies to embryos, our results provide direct evidence that X inactivation in preimplantation embryos occurs mainly, if not exclusively, in extraembryonic cells. This result is consistent with cell transplantation experiments which indicate that X inactivation in embryonic cells occurs after implantation (Gardner and Lyon, 1971). X-MCBs were first seen between the 8- and 16-cell stages, shortly after differentiation of trophectoderm begins. Early signs of trophectoderm differentiation begin after compaction of the 8-cell embryo, when changes are first observed in proteins involved in cell polarity (Hyafil et al., 1980; Ziomek and Johnson, 1980) and intercell contact, such as tight junction-specific proteins (Fleming et al., 1989). By the 32-cell stage, X-MCBs were present in the majority of cells in the embryos that contained X-MCBs (for example, see 27-cell embryo in Fig. 2).

In late morulae and early blastocysts, X-MCB-containing cells were not confined to the outer layer of cells. This appears to be consistent with the idea that some cells which become trophectoderm initiate differentiation while they are in the interior region of the embryo (Gardner, 1996; but see Winkel and Pedersen, 1988). The small number of X-MCB-containing cells associated with the ICM in later blastocysts were often adjacent to the blastocoel, suggesting that they could be, or will become, primitive endoderm, an extraembryonic lineage that forms a layer on the inner surface of the inner cell mass. These cells begin to differentiate prior to implantation (Gardner,

1983) and appear to initiate X inactivation at the late blastocyst stage (Tam et al., 1994).

The accumulation of macroH2A1.2 on one X chromosome occurs early in the progression of imprinted X inactivation. The precise timing of the initiation of imprinted X inactivation has not been determined. The activities of X-linked enzymes (Adler et al., 1977; Epstein et al., 1978; Kratzer and Gartler, 1978; Monk and Kathuria, 1977) and the relative concentrations of allele-specific RNAs (Singer-Sam et al., 1992) indicate that both X chromosomes of female mouse embryos are active at the 8-cell stage, and that inactivation has occurred in most cells by the blastocyst stage. The presence of X-MCBs in early morulae suggests that the accumulation of macroH2A1.2 on the X chromosome occurs before or possibly coincident with the initiation of transcriptional silencing.

Of the known features that distinguish the inactive X chromosome, namely the accumulation of *Xist* RNA, asynchronous replication, hypermethylation of CpG islands and histone hypoacetylation, only the accumulation of macroH2A1 has been shown to occur during the developmental time period when imprinted X inactivation is initiated in preimplantation embryos. Asynchronous replication of the inactive X chromosome was not observed until the late blastocyst stage, and does not appear to be occurring in the majority of cells at that stage (Sugawara et al., 1985). Inactive X-specific DNA methylation patterns also seem to occur too late to be involved in the initiation of imprinted inactivation, occurring only after implantation for *HPRT* (Lock et al., 1987) and *G6pd* (Grant et al., 1992). Methylation of *Pgk-1* begins earlier, with a low level detected in 40% of samples analyzed from blastocysts (Grant et al., 1992). On the basis of these data the accumulation of macroH2A1.2 on the X chromosome precedes changes in replication timing or inactive X-specific DNA methylation and, therefore, does not appear to be a secondary effect dependent on either of these changes. To our knowledge the status of histone acetylation during imprinted X inactivation has not been reported. The accumulation of *Xist* RNA appears to precede X inactivation in preimplantation embryos, since *Xist* RNA from the paternal X chromosome can be detected in 4-cell and 8-cell embryos (Kay et al., 1994). FISH has shown that the nuclei of all cells of 8-cell embryos have a large domain of *Xist* RNA similar to what is seen in somatic cells that have an inactive X chromosome (Sheardown et al., 1997). Thus, although expression of *Xist* seems to be required for the initiation of X inactivation, it does not appear to be sufficient by itself to bring about transcriptional silencing. The timing of macroH2A1 accumulation suggests that this process could be involved in initiation of imprinted X inactivation.

The sequence of events in the early stages of X inactivation in XX-ES cells is not the same as in preimplantation embryos. While macroH2A1 accumulation is an early event in preimplantation embryos, it occurs about 5 days after initiation of X inactivation in cultured XX-ES cells (Mermoud et al., 1999). On the other hand, asynchronous replication is an early event in XX-ES cells, but occurs after X inactivation in preimplantation embryos. It is not clear if these differences reflect intrinsic differences between imprinted and random X inactivation or changes that result from the culture of ES cells in vitro. *Xist* is required for initiation in both ES cells (Penny et al., 1996) and embryos (Marahrens et al., 1997), and

expression of stable *Xist* transcripts appears to occur before inactivation in both systems (Panning et al., 1997; Sheardown et al., 1997). One explanation for the difference between these systems is that *Xist* is capable of initiating inactivation by more than one mechanism, e.g. by causing asynchronous replication or macroH2A1 accumulation. Each of these mechanisms may by itself be capable of initiating transcriptional silencing. Later in development other mechanisms such as DNA methylation and histone hypoacetylation are added to ensure the long-term maintenance of silencing. It is also possible that both XX-ES cells and preimplantation embryos share a common but unidentified mechanism of initiating X inactivation.

We previously suggested that *Xist* RNA could play a role in localizing macroH2A1 to the inactive X chromosome (Costanzi and Pehrson, 1998; Pehrson and Fuji, 1998). This view is supported by results in mouse fibroblasts that showed that localization of macroH2A1 to the inactive X chromosome is lost when *Xist* was mutated (Csankovszki et al., 1999). Our present results are consistent with this possibility, in that they indicate that macroH2A1 accumulation in the inactive X chromosome occurs after *Xist* RNA accumulation, but before other known changes in the inactive X. However, MCBs were not seen in 8-cell embryos and occurred in only some cells at the 16-cell stage, despite the apparent presence of *Xist* RNA (Sheardown et al., 1997) and macroH2A1 in all cells of 8- and 16-cell embryos. This suggests that factors other than the presence of macroH2A1 and *Xist* RNA are involved in initiating the formation of X-MCBs. An increase in the concentration of macroH2A1 during these stages could be a factor, but we did not observe a consistent difference in the intensity of macroH2A1.2 immunofluorescence between cells that had an MCB and those that did not. Other possible changes that could trigger MCB formation include changes in the structure or localization of *Xist* RNA, post-translational modification of macroH2A1, removal of inhibitory factors, or induction of other required protein(s) or RNA(s).

In summary, our results clearly establish that histone macroH2A1 accumulates in the inactive X chromosome in mouse preimplantation embryos. This accumulation occurs predominantly, if not exclusively, in the extraembryonic lineages in blastocysts, and is first seen in morulae around the time when these cells are beginning to differentiate and initiate imprinted X inactivation. This early timing suggests that macroH2A1 accumulation does not depend on global changes in X chromosome replication timing or DNA methylation patterns, but leaves open the possibility of a relationship between *Xist* and macroH2A1 accumulation. Of the changes that are known to happen during X inactivation in preimplantation embryos, the accumulation of macroH2A1 appears to be the earliest marker of the inactive X chromosome and is the only change that has been shown to occur during the period when transcriptional silencing is initiated.

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