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Developmental and Tissue Expression Patterns of Histone MacroH2A1 Subtypes

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Abstract MacroH2A is a novel nucleosomal core histone that contains a large nonhistone region and a region that closely resembles a full length histone H2A. We have cloned a cDNA that contains the entire coding region of macroH2A1.2, one of the two identified subtypes of macroH2A1. MacroH2A1.2 was found to differ from the other known subtype, macroH2A1.1, in a single segment of the nonhistone region. MacroH2A1 specific antibodies revealed relatively high levels of both subtypes in adult liver and kidney. MacroH2A1.1 was much lower in fetal liver and kidney in comparison to their adult counterparts, and was not detected in adult thymus and testis, tissues with active cell division and differentiation. Both subtypes were present at very low levels or absent from mouse embryonic stem cells maintained in an undifferentiated state by growth in the presence of leukemia inhibitory factor. MacroH2A1.2 increased when the embryonic stem cells were induced to differentiate in vitro, while macroH2A1.1 remained undetectable. These results support the idea that macroH2A1.1 and macroH2A1.2 are functionally distinct, and suggest that changes in their expression may play a role in developmentally regulated changes in chromatin structure and function. J. Cell. Biochem. 65:107–113.

Key words: chromatin; nucleosome

The nucleosome consists of about 200 bp of DNA associated with an octameric complex of the four core histone proteins H2A, H2B, H3, and H4, and usually, one molecule of histone H1. The high evolutionary conservation of this basic structural unit of chromatin is consistent with its known roles in DNA packaging and regulation of gene expression [Felsenfeld, 1992]. A study to identify proteins that may be involved in modifying nucleosome structure and function led to the discovery of a new type of core histone, macroH2A (mH2A), in rat liver nucleosomes [Pehrson and Fried, 1992]. It is three times the size of a conventional H2A, and has a hybrid structure consisting of a large nonhistone region and a region that closely resembles a full length H2A. In rat liver it was estimated that there is approximately one mH2A1[1] for every 30 nucleosomes [Pehrson and Fried, 1992].

Two distinct mH2A1 cDNAs have been identified [Pehrson and Fried, 1992]. One contained a complete coding region and was named mH2A1.1, while the other was produced by PCR and was incomplete. The nucleotide sequences of the cDNAs were identical to each other except for one region. The region of nonidentity occurred in a part of mH2A1 that resembles a leucine zipper, a helical structure involved in protein-protein interactions through the formation of a coiled-coil [Landschulz et al., 1988; Oshea et al., 1991]. Within this region, both mH2A1 subtypes have sequences consistent with a coiled-coil interaction. However, they differ at sites that could affect the specificity of such an interaction, suggesting that they may be functionally distinct.

The unique structure of mH2A suggests that nucleosomes containing it have functions that

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[1]We have discovered a second mH2A gene which we call mH2A2. The mH2A subtypes described in this paper and in Pehrson and Fried (1992) are from the mH2A1 gene, and are referred to as mH2A1.1 and mH2A1.2.

are distinct from other nucleosomes. One example of a structurally diverged core histone with a specialized function is H2A.Z, a subtype of H2A that is essential in drosophila [van Daal et al., 1992]. It constitutes approximately 5% of the total H2A in the vertebrate tissues and cells that have been examined [West and Bonner, 1980] and is present at an essentially constant level at all stages of drosophila development [van Daal et al., 1992]. This suggests that H2A.Z is involved in a chromatin function that is required in most cells and developmental stages. MacroH2A1, on the other hand, could be involved in a chromatin function that is different in different cell types and/or changes during development. In this case, the mH2A1 content and/or subtype composition of different tissues is expected to be different and to change during development. Therefore, information about the pattern of mH2A1 expression should be useful for assessing possible functions, and possible distinctions between the subtypes. In this paper we report the cloning and sequencing of a cDNA that contains the complete coding region of the second mH2A1 subtype, mH2A1.2. We also describe the use of mH2A1 specific antibodies to examine the expression of mH2A1.1 and mH2A1.2 in different mammalian tissues and at different stages of development.

MATERIALS AND METHODS

Cloning and Sequencing of mH2A1.2

A rat thymus, oligo dT primed cDNA library in λZAPII was propagated in LE392 host bacteria. Plaque lifts were screened with an amplified fragment from the incomplete PCR generated cDNA clone [Pehrson and Fried, 1992]. Positive secondary clones were excised in vivo using R408 helper phage. The plasmid clones were sequenced using T3, T7, and internal primers [Sanger et al., 1977]. The sequence of the rat mH2A1.2 cDNA was submitted to Genbank, accession number U79139.

Production and Purification of Antibodies

The peptides used for antibody production were synthesized using an Applied Biosystems model 431A peptide synthesizer. The peptide sequences are: 1: CNTDFYIGGEV (from the region of nonidentity of mH2A1.1), 2: CNADIDLKDDL (from the region of nonidentity of mH2A1.2), and 3: CYVQEMAKLDAN (from the C-terminus). The cysteine on the N-terminus of each peptide is not present in mH2A1 and was added to simplify their conjugation to bovine serum albumin. The conjugation was done using the crosslinker maleimidobenzoyl-N-hydroxylsuccinimide ester [Harlow and Lane, 1988]. Antibodies against the conjugates were raised in chickens and IgY was purified from egg yolks [Gassmann et al., 1990]. The anti-peptide antibodies were purified at room temperature by passing the total IgY through a column containing the peptide immobilized on Affi-Gel 10 (BioRad) agarose beads; approximately 1 mg of peptide was conjugated per ml of beads. Bound antibodies were eluted with 6 M guanidine chloride, 10 mM Tris pH 7.5. Antibodies were renatured at room temperature by dialysis against 10 mM Tris pH 7.5, 150 mM NaCl (TBS). Dialysis was done stepwise doubling the volume of TBS every 20 min. After the fourth step the sample was dialyzed overnight at 4°C against 4 liters of TBS.

Western Blots of Nuclear Extracts

Frozen tissues from mature and fetal animals were obtained from Pel-Freez Biologicals, except for fetal mouse liver which was provided by David Goldhamer (University of Pennsylvania). Nuclei were purified essentially as previously described [Pehrson, 1989]. Two methods of nuclear extraction were compared for their efficiency of mH2A1 extraction. One involved digestion of the nuclei with micrococcal nuclease followed by addition of an equal volume of 2X SDS sample buffer to the total digest and brief heating to 100°C. In the second method nuclei were extracted twice with 0.4 N H_2SO_4 and the extracted proteins were precipitated by adding trichloroacetic acid to 20%. The precipitate was washed once with ethanol containing 0.2% H_2SO_4, twice with ethanol, dried, and dissolved in SDS sample buffer. The ratio of mH2A1 to the other core histones was essentially the same for both methods. H_2SO_4 extracts were used for the blots in this paper since they produce cleaner extracts. The extracts were run in 12.5% polyacrylamide (1:125 bisacrylamide:acrylamide) SDS gels [Laemmli, 1970]. Loadings were adjusted to equalize the amount of core histone in each lane. The proteins were transferred from the gel onto polyvinylidene difluoride membranes in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid pH 11 [Matsudaira, 1987]. The membranes were blocked for 2 h at room temperature with TBS containing

5% nonfat dry milk and then were reacted with the anti-peptide antibodies in the same solution overnight at 4°C. They were washed three times with TBS containing 5% milk and then reacted with alkaline phosphatase conjugated rabbit anti-chicken IgG (Sigma, St. Lious, MO) for 1 h at room temperature. The membranes were washed twice with TBS containing 5% milk, twice with TBS, and developed using an alkaline phosphatase substrate kit (Pierce, Rockford, Illinois).

Culture of Embryonic Stem Cells

Mouse embryonic stem (ES) cell line D3 was obtained from American Tissue Type Collection (ATCC CRL 1934). The cells were cultured in the absence of feeder cells on gelatin coated tissue culture dishes, in DMEM supplemented with 15% fetal calf serum, 2 mM glutamine, 100 mM β-mercaptoethanol, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Leukemia inhibitory factor (LIF) was supplied by the addition of media conditioned by CHO cell line 8/24 720 LIF-D(.1) (Genetics Institute, Cambridge, MA). The conditioned media was added to a final concentration of 5%. When grown in the LIF containing media the cells grew rapidly as tightly clustered groups of cells with a morphology essentially identical to that published for undifferentiated ES cells [Williams et al., 1988]. Differentiation was initiated by culturing trypsinized cells on petri dishes in the standard medium without the LIF conditioned medium. Under these conditions the cells formed embryoid bodies [Doetschman et al., 1985]. After about 5 days the embryoid bodies were transferred to gelatin coated culture dishes and growth continued in the absence of LIF. As previously reported [Doetschman et al., 1985], several different cell morphologies were observed in these differentiating cultures.

RESULTS

Cloning and Sequencing of a cDNA Encoding mH2A1.2

A rat thymus cDNA library was screened using a fragment from the nonhistone region of mH2A1. Three clones were identified and partial sequences from the ends of one clone showed that it contained the entire coding region. Complete sequencing of this clone revealed it to be identical to mH2A1.1 in all regions except one. In this region the new clone is identical to the partial cDNA originally amplified by PCR from a rat thymoma cDNA library [Pehrson and Fried, 1992]. We named the subtype encoded by this cDNA mH2A1.2. Figure 1 diagrams the relationship between mH2A1.1, mH2A1.2, and the original partial cDNA amplified by PCR.

Detection of mH2A1 Subtypes With Anti-Peptide Antibodies

Antibodies were raised against peptides that had sequences based on different regions of mH2A1. Three peptides produced antibodies that reacted with mH2A1 on Western blots. The positions of these peptide sequences in mH2A1 are shown in Figure 1. Two of these peptides, 1 and 2, were based on the region of nonidentity of mH2A1.1 and mH2A1.2 respectively. Peptide 3 was based on the C-terminal sequence which is common to both subtypes. The anti-sera against these peptides were used to stain Westerns blots of proteins extracted from nuclei isolated from adult rat liver, mouse liver, and rabbit kidney (Fig. 2). The antiserum against the peptide based on the C-terminal sequence reacted with two bands with electrophoretic mobilities identical to the two mH2A1 bands seen in purified rat liver chromatin fragments [Pehrson and Fried, 1992] (Fig. 2). The antiserum against the peptide specific to mH2A1.1 reacted only with the upper mH2A1 band and the antibody against the peptide specific to mH2A1.2 reacted only with the lower

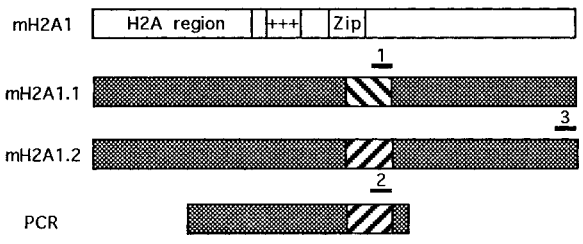

Fig. 1. Comparison of mH2A1.1 and mH2A1.2. A diagram of mH2A1 structure is shown on top. +++Indicates a lysine rich region that resembles part of the C-tail of histone H1, and Zip indicates a region that resembles a leucine zipper [Pehrson and Fried, 1992]. The relationship of mH2A1.1 and mH2A1.2 is shown below. The gray areas indicate regions that are identical in both amino acid and nucleotide sequences. The regions of nonidentity are indicated by the hatched areas. The amino acid sequences of these regions are only 24% identical. The short bars marked 1, 2, and 3 indicate the locations of the peptide sequences used to raise mH2A1 specific antibodies. The structure of the partial cDNA obtained by PCR amplification [Pehrson and Fried, 1992] is diagrammed at the bottom. The sequence of the rat mH2A1.2 cDNA was submitted to Genbank, accession number U79139.

mH2A1 band. Using the antiserum against the C-terminal peptide of mH2A1 we have also detected two bands of essentially identical mobility in human and feline liver (data not shown).

Expression of mH2A1 Subtypes in Different Tissues

The peptide specific antibodies were used to examine the mH2A1 content of tissues from rats, mice, and rabbits. For this purpose, Western blots of nuclear extracts separated by SDS polyacrylamide gel electrophoresis were stained with the antiserum against the C-terminal peptide. On the basis of the staining pattern obtained with extracts of purified rat liver chromatin fragments, where the two mH2A1 bands can be seen by staining with coomassie blue, the antiserum against the C-terminal region appears to react equally well with mH2A1.1 and mH2A1.2.

Rat liver was found to contain nearly equal amounts of mH2A1.1 and mH2A1.2 (Fig. 3A). Rat brain had a relatively high level of mH2A1.2, and a low level of mH2A1.1. By comparison, the mH2A1 contents of rat thymus and testis were lower and only mH2A1.2 was detected. The mH2A1 staining pattern of independent preparations from the same tissue were similar indicating that these results reflect stable differences between these tissues. A similar pattern was seen with mouse and rabbit tissues (Fig. 3A). Mouse liver and kidney, and rabbit kidney contained both mH2A1.1 and mH2A1.2, while mouse thymus and testis, and rabbit testis contained only mH2A1.2.

Since the antiserum used in these studies should only react with a short region in the C-terminus of mH2A1, it was possible that post-translational modification of this region could

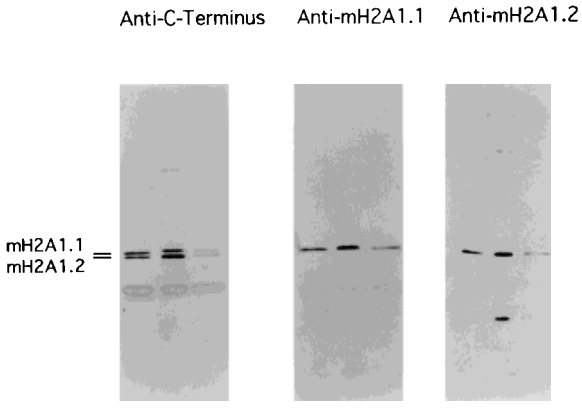

Fig. 2. Identification of mH2A1.1 and mH2A1.2 with mH2A1-specific antibodies. Western blots with antibodies against the C-terminus of mH2A1, or the region of nonidentity of mH2A1.1 or mH2A1.2, were used to detect mH2A1 in nuclear extracts from **(1)** rat liver, **(2)** mouse liver, and **(3)** rabbit kidney. The fainter bands below the mH2A1 bands in the blot stained with the antibodies against the C-terminus are histone H1, which cross-reacts weakly with this antibody for unknown reasons. The antibody against the region of nonidentity of mH2A1.2 reacts with an unknown lower molecular weight protein.

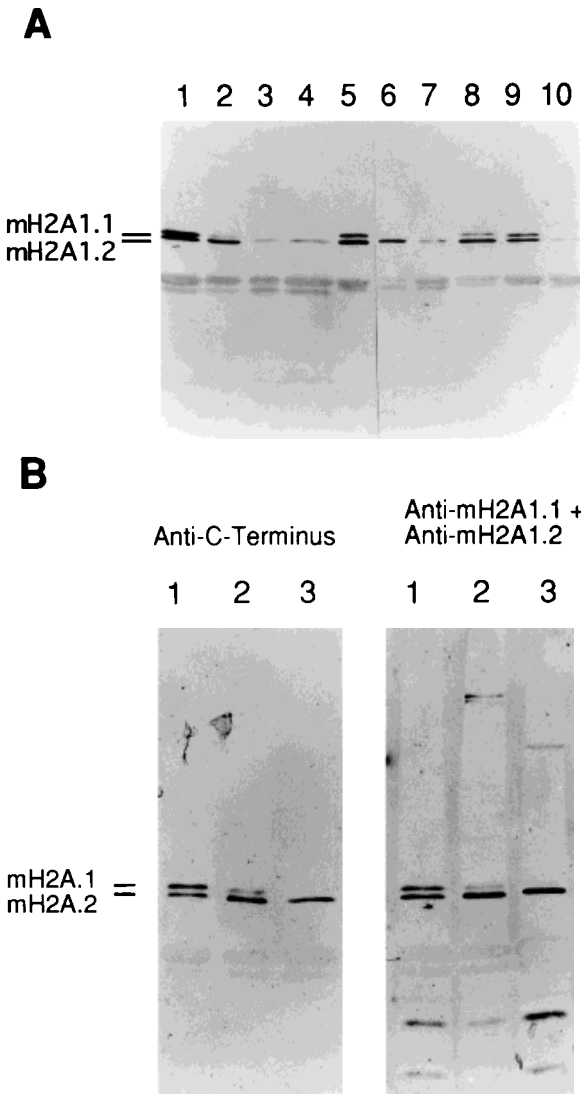

Fig. 3. The mH2A1 content of different tissues: **A:** Western blot with antibodies against the C-terminus of mH2A1. Nuclear extracts were prepared from: **(1)** rat liver; **(2)** rat brain; **(3)** rat testis; **(4)** rat thymus; **(5)** mouse liver; **(6)** mouse testis; **(7)** mouse thymus; **(8)** mouse kidney; **(9)** rabbit kidney; **(10)** rabbit testis. Staining of core histones with coomassie blue was used to equalized loadings. **B:** Comparison of mH2A1 staining patterns obtained with antibodies against the C-terminus to that obtained with a mixture of antibodies against the regions of nonidentity of mH2A1.1 and mH2A1.2. Nuclear extracts were from: **(1)** rat liver, **(2)** rat brain, and **(3)** rat thymus.

interfere with antibody binding. Thus, the tissue differences in the staining of mH2A1 subtypes we observed with the antiserum against the C-terminus could reflect cell type restricted post-translational modification rather than different mH2A1 contents. This possibility was addressed by staining extracts from rat liver, brain, and thymus with a mixture of the two anti-sera against the regions of nonidentity. This mixture of anti-sera should stain both subtypes, but by binding a totally different region of mH2A1. The relative staining of mH2A1.2 by this mixture was greater than that of mH2A1.1, because of a difference in the titers of the two anti-sera. However, the relative staining of the mH2A1 subtypes in different tissues was very similar to that obtained with the antiserum against the C-terminus (compare Fig. 3B to C). This result indicates that the tissue differences we have observed reflect protein content, rather than protein modification.

Expression of mH2A1 Subtypes at Different Stages of Development

In order to examine whether the pattern of mH2A1 subtypes present in a given tissue changes during development we examined the mH2A1 content of fetal liver and kidney. Nuclear extracts from 20-day fetal rat liver and kidney, and 16-day fetal mouse liver were examined on Western blots using antibodies against the C-terminus (Fig. 4A). In contrast to their adult counterparts all three fetal tissues contained predominantly mH2A1.2 and very little mH2A1.1.

To investigate mH2A1 expression during earlier stages of development we examined the expression of mH2A1 subtypes in mouse embryonic stem cells (ES cells). ES cells are derived from the inner cell mass of the blastocyst stage of development. They can be grown in vitro and maintained in a totipotent state by the addition of leukemia inhibitory factor (LIF) to the media [Williams et al., 1988; Smith et al., 1988]. When grown in the absence of LIF they differentiate into several different cell types [Doetschman et al., 1985]. Thus, ES cells make a good model system for examining early stages of mammalian differentiation.

The expression of mH2A1 subtypes in ES cells grown in the presence, or absence of LIF was analyzed on Western blots stained with antibodies against the C-terminus (Fig. 4B). In comparison to mouse liver, the expression of both mH2A1 subtypes was very low in undifferentiated cells. The antibodies reacted with an unknown protein of electrophoretic mobility slightly faster than mH2A1.2. This protein did not react with either of the subtype specific antibodies, indicating that it is not a modified form of these mH2A1 subtypes. Although both mH2A1.1 and mH2A1.2 were virtually undetectable in the undifferentiated ES cells used in this experiment, very low levels of mH2A1.2 were detected in a different ES cell line. Low, but easily detectable levels of mH2A1.2 were observed when ES cells were grown in the absence of LIF (Fig. 4B). To confirm that the band we were detecting was mH2A1.2, we also stained Western blots of the same extracts with the anti-serum that reacts with the region of nonidentity of mH2A1.2 (Fig. 4C). We did not detect mH2A1.1 in these extracts.

DISCUSSION

We examined the expression of mH2A1 subtypes in different tissues, and at different developmental stages, in order to gain insights into possible function(s) for these proteins. These studies show that mH2A1 is not a constitutive component of the chromatin, but rather, varies both in total amount and subtype composition. We found that mH2A1 can vary from a level where both subtypes are undetectable or barely detectable with our antibodies, to the relatively high level of both subtypes that is present in adult liver.

The expression patterns for mH2A1.1 and mH2A1.2 were distinctly different. Substantial levels of mH2A1.1 were found only in adult tissues that have few or no dividing cells. In such tissues its level was found to be variable, being relatively high in liver and kidney, and lower in brain. These data indicate that the accumulation of mH2A1.1 in the chromatin may be related to the cessation of cell division and/or late stages of differentiation. The very low level of mH2A1.1 in fetal liver, relative to adult liver, does not appear to be related to hematopoiesis, because the fetal rat livers were obtained from animals at a developmental stage where hematopoiesis in the liver has essentially ceased [Bankston and Pino, 1980].

In contrast to mH2A1.1, mH2A1.2 is present at a substantial level in tissues with extensive cell proliferation. This includes differentiating ES cells, fetal liver and kidney, and adult thy-

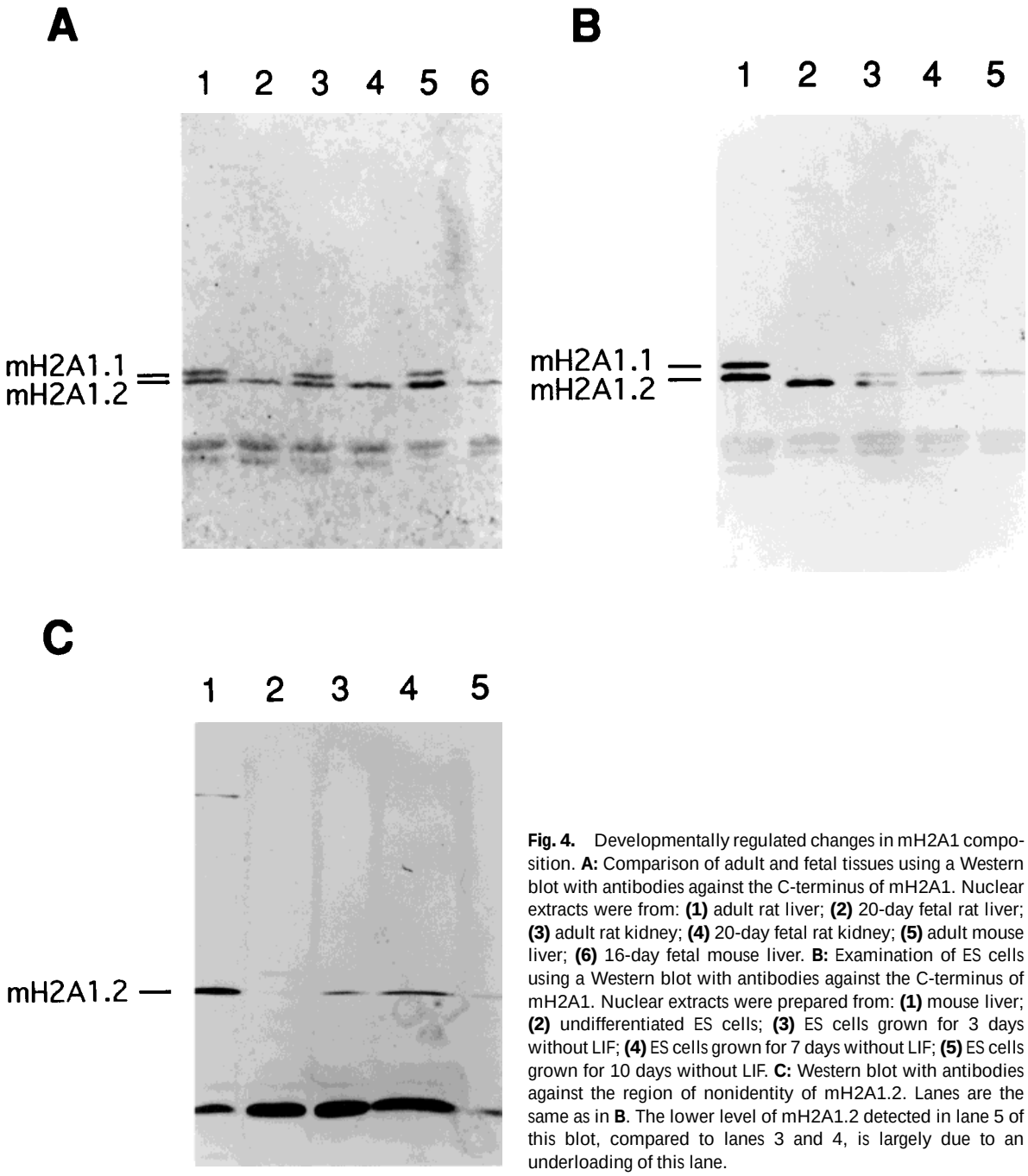

Fig. 4. Developmentally regulated changes in mH2A1 composition. **A:** Comparison of adult and fetal tissues using a Western blot with antibodies against the C-terminus of mH2A1. Nuclear extracts were from: **(1)** adult rat liver; **(2)** 20-day fetal rat liver; **(3)** adult rat kidney; **(4)** 20-day fetal rat kidney; **(5)** adult mouse liver; **(6)** 16-day fetal mouse liver. **B:** Examination of ES cells using a Western blot with antibodies against the C-terminus of mH2A1. Nuclear extracts were prepared from: **(1)** mouse liver; **(2)** undifferentiated ES cells; **(3)** ES cells grown for 3 days without LIF; **(4)** ES cells grown for 7 days without LIF; **(5)** ES cells grown for 10 days without LIF. **C:** Western blot with antibodies against the region of nonidentity of mH2A1.2. Lanes are the same as in **B**. The lower level of mH2A1.2 detected in lane 5 of this blot, compared to lanes 3 and 4, is largely due to an underloading of this lane.

mus and testis. Its presence during early stage of ES cell differentiation indicates that it begins to accumulate at very early stages of development. From our data we cannot tell whether it is absent from undifferentiated ES cells, or present at a much reduced level. The mH2A1.2 level is as high or higher in adult liver and kidney than it is in embryonic or fetal tissues. Thus, the role of mH2A1.2 would not appear to be specific for dividing or differentiating cells. Also, it does not appear that mH2A1.1 replaces mH2A1.2 in the chromatin, because there is no apparent drop in mH2A1.2 with the accumulation of mH2A1.1 in adult liver and kidney.

Previously, it was estimated that there is approximately one mH2A1 molecule for every 30 nucleosomes in adult rat liver [Pehrson and Fried, 1992]. Our present studies have not found significantly higher levels in other tissues, or in early development. If mH2A1 is distributed

evenly throughout the genome, its low level relative to the other histones suggests a role involving relatively large segments of chromatin. Such a function could involve interactions within or between chromatin fibers, or an interaction between chromatin and other nuclear structures. Alternatively, mH2A1 might be localized to specific regions of chromatin, where it could help produce specialized chromatin structures or nucleosome arrangements. One interesting possibility is that mH2A1 plays a role in developmentally regulated changes in chromatin structure related to changes in gene expression and/or cell proliferation.

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