Developmental expression of H3.3A variant histone mRNA in mouse

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ABSTRACT Cloning and characterization of H3.3A variant histone expression has recently been reported to be associated with meiotic development in mouse testis and ovary. Using Northern analysis and in situ hybridization, the pattern of H3.3A expression was studied during the development of different tissues. In addition to the differential expression detected in male and female meiosis, H3.3A was found to be highly expressed in preantral follicles of adult ovaries and in the basal regions of seminiferous epithelium corresponding to spermatogonia. Different patterns of expression were observed in somatic tissues, which also differed with respect to the developmental stage of the tissue. The lowest expression was detected in adult skeletal muscle. High expressions were found in foetal liver and spinal cord. These different expressions might reflect a possible function of H3.3A in cell differentiation as detected in MEL cells.

KEY WORDS: H3.3A, variant histone, gene expression

Introduction

Eukaryotic histone genes are grouped into three distinct categories: a) replication-dependent histones, whose expression is restricted to the S-phase of the cell cycle and which comprise the “core histones” H2a, H2b, H3, H4, and the “linker histone” H1; b) replacement variant histones, encoded by genes that are constitutively expressed in proliferative and quiescent cells, and during different stages of differentiation and c) tissue-specific histones, whose expression is confined exclusively to defined tissues. Unlike the particular features observed in the replication-dependent histones (intron-less, poly[A]̇), variant histone genes have introns and encode polyadenylated transcripts. Despite the fact that histones are very conserved proteins, the functions of variant histones are still unknown, though they have frequently been described as involved with the processes of cell differentiation (Marzluff, 1989). The variant histones include the vertebrate H3.3 histones. These are coded by two different genes, H3.3A and H3.3B, which generate similar, conserved proteins from transcripts differing in their 5’ and 3’ untranslated regions. In the mouse, H3.3B cDNA and pseudogenes have been reported (Wellman et al., 1987; Hraba-Renevey and Kress, 1989). We have recently cloned and characterized a mouse cDNA for H3.3A variant histone, and demonstrated that H3.3A variant histone cDNA contains an unusual α-globin insertion in the 5’ UTR. Its expression is modulated during gametogenesis and is associated with the onset of meiosis. (López-Alañón et al., 1997)

In the present study, in situ hybridization and Northern blot analysis were used to characterize H3.3A expression during gametogenesis in male and female gonads and in somatic tissues at different stages of development. The differential patterns observed in the various tissues analyzed add to our understanding of a possible role of H3.3 histones in cell differentiation.

Results

Expression of H3.3A during development of the ovary

In foetal ovaries, the pattern of gene expression for H3.3A histone reveals a decrease in transcript accumulation from day 13 postcoitum (pc) to birth (López-Alañón et al., 1997). In situ hybridization experiments, using antisense riboprobes of a Kpn1-Xhol fragment specific to H3.3A 3’UTR (see Materials and Methods and Fig. 1), displayed a speckled hybridization pattern in foetal ovary sections that could correspond to a dispersed distribution of oocytes in the foetal ovary (not shown). Nevertheless, in adult ovaries, a higher level of signal accumulation was observed in preantral follicles (Fig. 2). To determine whether these signals were due to a higher cell density, a comparison was made between

Abbreviations used in this paper: MEL, murine erythroleukemia; UTR, untranslated region; pc, postcoitum; pn, postnatal; DME, Dulbecco modified eagle; HMBA, hexamethylenesacacetamide; UV, ultraviolet; SDS, lauryl sodium sulphate; PBS, phosphate buffer saline.
Expression of H3.3A in developing testis

In mammalian testis, spermatogenesis progresses after a proliferative phase of gonocytes. G1 spermatogonia become type A spermatogonia (Bellvé et al., 1977). The first preleptotene spermatocytes appear in the mouse at about day 10 of postnatal life (pn). Northern analysis performed on testis at different stages of development revealed an increase in H3.3A expression at day 6 pn, with a prominent peak at day 10 pn. This corresponds to the beginning of meiosis (López-Alañón et al., 1997).

In situ hybridization with an antisense riboprobe showed a widespread signal in adult testis sections (Fig. 4 A). However, a stronger signal at the basal region of the seminiferous epithelium was detected (Fig. 4 C). This was improved when the emulsion used for in situ preparations was exposed for less time (Fig. 4 B). This pattern correlates with the increase in gene expression observed in prepuberal animals at day 10 pn. Meiosis starts in type B spermatogonia which are located on the basal layer of the seminiferous tubule epithelium.

Gene expression in somatic tissues

In order to analyze H3.3A expression during development of somatic tissues, Northern blot analysis was performed using total RNA isolated from different tissues of foetal and adult mice. MEL cells provide a useful model for studying differentiation and were used here as a control of the level of H3.3A expression. These cells are blocked at the proerythroblast stage of erythroid maturation. Several compounds, such as HMBA, can induce the cells to overcome the block and reinitiate the differentiation programme. Changes in the expression of several genes, including H3.3A, have been described as early events that may influence commitment of MEL cell differentiation (Krimer et al., 1993). Series of Northern blots using MEL cells, either untreated or treated with HMBA from 4 to 72 h (Fig. 5), were hybridized with a 440 bp Styl-Xhol fragment, as a specific probe, from the 3' end UTR of the H3.3A cDNA (Fig. 1). The pattern of mRNA accumulation during MEL differentiation was similar to that previously described using an H3.3A genomic pseudogene probe (Krimer et al., 1993). A similar amount of total RNA isolated from adult testis was loaded to compare the expression of the variant histone in both MEL cells and testis. The level of expression observed in testis was at least twice the maximum signal (8 and 12 h after induction) detected in MEL cells committed to differentiation.

Northern analysis of H3.3A in a variety of foetal and adult somatic tissues revealed a single 1.3 Kb transcript similar to that observed in gonadal tissues and MEL cells (Fig 6). However, the level of expression varied among the different tissues. Differences
Fig. 3. Image analysis of signal obtained by in situ hybridization in sections of adult ovaries. (A) Control of density of cells by image analysis of histological preparations after haematoxylin/eosin staining. Arrows indicate antral follicles. (B) Distribution of radioactive signal after in situ hybridization with a specific H3.3A riboprobe. Gradation of the signal is shown in the right panel, which includes a scale of relative values.

were also seen depending on the developmental stage considered: foetal or adult. The lowest signal was detected in adult mouse skeletal muscle whilst the highest was found in foetal liver (Fig. 6). These results were clearly confirmed by in situ hybridization analysis in sections of embryos at day 13 pc. (Fig. 7). In addition, an in situ analysis of whole embryos showed high levels of expression in spinal cord and liver. Both differential expressions were better seen using the described image analysis procedure when cytological staining was compared with autoradiographic signal detected (Fig. 8).

Discussion

The high level of conservation of histones, including variant histones, reflects a possible, important role for these proteins during the cell cycle and differentiation. The high number of pseudogenes and genomic rearrangements observed in the histone family is also a clear indication of the evolutionary pressure it has faced. In fact, H3.3 histones could be considered one of the most conserved proteins in the animal kingdom (Akmanova et al., 1995). Such conservation of these genes, expressed from Droso phila (Akmanova et al., 1995) to mice, strongly suggests an important function in development and differentiation. The present study shows that H3.3A is widely expressed in different tissues. However, there are remarkable differences in the level of expression between tissues and throughout their development.

The participation of the H3.3 histone genes, H3.3A and H3.3B, in differentiation processes has been documented (Krimer et al., 1993; Castiglia et al., 1994). The expression pattern of the H3.3A histone gene during the differentiation of MEL cells committed to erythroleukemia (Krimer et al., 1993) was used to assess H3.3A expression during development and differentiation. The accumulation of H3.3A transcripts detected by Northern analysis in liver, gonads and muscle, confirmed differential expression of H3.3A variant histone in association with development and cell differentiation.

In order to estimate H3.3A expression in developing tissues previously analyzed by Northern blots, in situ hybridization was performed on whole embryo (13 days pc.) sections. The comparison of autoradiographic signals with photodensitometry data corresponding to cell concentrations in different tissues, provided confirmation of the high expression seen in foetal liver. The work of Krimer, et al. (1993) and the Northern blots of the present work, led these results to be expected. However, an unexpectedly high level was detected in spinal cord. At present, the functional

Fig. 4. Expression of H3.3A in seminiferous tubules. In situ detection of H3.3A transcripts in seminiferous epithelium. Sections of adult testis showing: (A) a speckled distribution in the whole tubule, (B, C) a more concentrated signal in the basal regions of testis tubules (arrows), especially well detected (B) when autoradiographic emulsions were exposed for less time. (A and B) Dark field illumination plus Hoechst staining. (C) Dark field. (D) Hoechst staining of (C) preparation.
Liver  Muscle  Kidney  Ovary  Testis

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<th>MEL cells in H MBA (hours)</th>
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<td>0  4  8  12  24  48  72</td>
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Fig. 5. H3.3A expression in differentiating MEL cells. Northern analysis of total RNA was performed using a specific probe for H3.3A. Samples were obtained from untreated or HMBA-treated MEL cells at different times. The last lane corresponds to a similar load of 15 mg of total RNA from adult testis.

significance this might have in the nervous system during this stage of development is unknown.

Using a similar approach, an accumulation of H3.3A transcripts was also detected in pre-antral follicles in adult ovary. This accumulation in somatic cells of follicles seems not to be associated to the putative role of H3.3 during early meiosis. However, in situ analysis of adult testis confirmed the suggested participation of H3.3A in the onset of meiosis (López-Álcan et al., 1997).

Fig. 6. Expression of H3.3A variant-histone mRNA from foetal and adult mouse tissues. Northern blot analysis of mouse RNA isolated from different tissues of foetal (F) and adult (A) animals. Blots were hybridized with an H3.3A cDNA probe and reprobed with a β-actin probe.

Fig. 7. Comparative levels of H3.3A transcript accumulation shown by in situ hybridization in lung (LU), heart (H) and liver (L) of a 13 day pc. embryo. Section hybridized with a riboprobe from 3'UTR of cloned H3.3A cDNA. (A) Hoechst staining. (B) Simultaneous Dark field and Hoechst staining. Note the greater labeling in liver.

The differential modulation of H3.3A and H3.3B expression during development and differentiation is not yet understood. Considering that these two genes code for the same H3.3 polypeptide (with the
exception of an arginine residue in H3.3B that changes to cysteine in H3.3A (López-Alaño et al., 1997), the specific differential regulation of these genes merits further investigation.

Materials and Methods

Clones and probes

A cDNA clone for the H3.3A variant histone was selected from a cDNA library prepared from mouse embryo ovary mRNA (16 days pc) (López-Alaño et al., 1997) (EMBL/GenBank Data Libraries under Accession No.X91866). The initial clone was isolated by differential screening of this library with single stranded cDNA probes from testis versus somatic probes (López-Alaño and del Mazo, 1995).

MEL cell culture

Line DS19 MEL cells were cultured in Dulbecco’s Modified Eagle’s medium (DME) supplemented with 10% (v/v) foetal bovine serum. Cells were induced to differentiate in the same media containing 5 mM hexamethylenesbenzacetamide (HMBA) as previously described (Krimer et al., 1993). The percentage of induced cells was determined by the benzidine staining reaction.

RNA isolation and analysis

Prenatal and postnatal mice were obtained from a Swiss inbred mouse colony. Tissue samples were dissected from embryos and adult animals. Total RNA was extracted from tissues and MEL cells by the acid guanidinium thiocyanate (Chomczynski and Sacchi, 1987).

Northern blots were prepared from total RNA. RNA was separated by electrophoresis under denaturing conditions in 1% (w/v) agarose with 8% (v/v) formaldehyde by loading agarose gels with 15 μg of RNA for each sample. RNAs were blotted onto nylon membranes by a vacuum blotter for 2 h and cross-linked by UV exposure. As a loading control, blots were reprobed with a β-actin probe. A specific 440 bp Sty1-XhoI fragment derived from the 3′ region of the H3.3A clone (López-Alaño et al., 1997)(Fig. 1) was used as a probe, labeled with [α-32P]dCTP by random hexanucleotide priming (Amersham). The filters were then hybridized at 42°C for 24-36 h and washed twice in 2xSSC, 0.1% (w/v) SDS for 15 min at room temperature, and three times in 0.1xSSC, 0.1% (w/v) SDS at 55°C for 10 min.

In situ hybridization

Adult tissues and 13 days pc. embryos were dissected and immediately frozen in dry ice. Sections of 12 mm were obtained in a cryostat, mounted on RNase-free slides coated with poly-L-lysine (50 mg/ml) and kept frozen at -80°C until use. The preparations were fixed with 4% (w/v) paraformaldehyde in PBS and processed as in López-Fernández, et al. (1995). Preparations were then hybridized with a 423 nucleotide riboprobe corresponding to the 3′ UTR of H3.3A (Fig. 1). Sense and antisense RNAs were obtained by in vitro transcription and labeled with [α-32P]UTP (Amersham). Hybridization conditions and stringency washes were the same as previously described.

After dehydration in ethanol, the slides were exposed in a cassette with Hyper film (Amersham) to control the signal level. After 3 or 4 days exposure, films were developed and the slides coated with LM emulsion (Amersham). These were then developed after exposure for the appropriate times and counterstained with 0.05 mg/ml Hoechst 33258 in the last waterwash (Thomas et al., 1990). Slides were then mounted, on glycerol:PBS (8:2), visualized with epifluorescence microscopy and photographed. Tissue morphology was observed and photographed using UV fluorescence and dark field illumination after staining the nuclei with Hoechst 33258 and detecting the radioactive signal with silver grains. The coverslips were removed by a water wash and the tissues dehydrated, stained with hematoxylin/eosin and permanently mounted on Entellan (Merck). Sense RNA probes were used as negative controls.

To discriminate signal accumulation in defined regions, both in adult ovaries and in whole embryos at 13 pc days, an image analysis procedure was performed. Both the hematoxylin/eosin stained preparations and the autoradiographic images on Hyperfilm were collected and processed quantitatively in a photodensitometer using an Image-Quant program. To enhance the differences, the images were transferred and processed using an NIH Image 1.52 program.

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