Comparative analysis of expression of histone H2a genes in mouse

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Abstract

Background: At least 18 replication-dependent histone H2a genes are distributed in 3 Hist gene clusters on different chromosomes of the mouse genome. In this analysis we designed specific PCR primers for each histone H2a transcript and studied the expression levels and patterns using quantitative RT-PCR (qRT-PCR). In addition, we compared histone H3 K9 acetylation levels in the promoter regions of H2a genes by ChIP (chromatin immunoprecipitation) – quantitative PCR (qPCR) analysis.

Results: RT-PCR analysis indicated that all 20 histone H2a genes assessed in this study are expressed. The replication-dependent histone H2a genes have different expression levels but similar expression patterns. Among the 20 histone H2a genes, the expression-level of H2afz, a replication-independent gene, was highest, and that of Hist1h2aa, a replication-dependent gene, was lowest. Among 18 replication-dependent H2a genes, the expression level of Hist3h2a was highest. The ChIP-qPCR analysis showed that histone H3 K9 acetylation levels in promoter regions of both H2afz and Hist3h2a are clearly higher than that in the promoter region of Hist1h2aa. The H3 K9 acetylation level in the promoter of Hist1h2aa is similar to that in the γ-satellite region.

Conclusion: These results strongly suggest that histone H3 K9 acetylation plays a role in the expression of histone genes.
structure. H3 K9 methylation is enriched in transcriptionally silent genes and heterochromatin. On the other hand, H3 K9 acetylation is enriched in transcriptionally active genes [7]. Is this modification related to histone gene expression?

Eighteen replication-dependent histone H2a genes were identified in the mouse genome sequence [8]. Among these 18 genes, 13 are located in the Hist1 cluster on chromosome 13, 4 in the Hist2 cluster on chromosome 3, and 1 in the Hist3 cluster on chromosome 11 [8]. Thus, replication-dependent histone H2a genes are distributed in at least 3 Hist clusters. In addition, the mouse has 2 replication-independent histone H2a genes, H2afx on chromosome 9 and H2afz on chromosome 3. Recently we reported a novel replication-independent histone H2a gene (H2afj) on chromosome 6 [9]. H2afz and H2afj are typical replication-independent genes [9,10]. The H2afz protein is enriched in euchromatic regions and acts synergistically with a boundary element to prevent the spread of heterochromatin [6]. On the other hand, H2afx mRNA has both a polyadenylated tail and a stem-loop structure [11], elements typical of, respectively, replication-independent and replication-dependent histone genes.

As cells progress from G1 to S phase, the rate of histone gene transcription increases 3- to 5-fold, and the efficiency of histone pre-mRNA processing increases 8- to 10-fold, resulting in a 35-fold increase in histone protein levels [2,12]. Most promoters of histone genes have CCAAT and TATA boxes [9,13]. Some promoters have an E2F binding motif between the CCAAT and TATA boxes. This E2F binding motif is recognized, and then the E2F transcription factor activates an H2a gene in early S-phase of the cell cycle [14]. However, it is not known how transcription-related proteins cooperate to coordinately regulate histone gene transcription during the cell cycle.

The amino acid sequences of histone H2a proteins are very similar, except for that of H2afz protein [9]. For example, Hist1h2ab, 2ac, 2ad, 2ae, 2ag, 2ai, 2an, and 2ao encode the same structural protein. Among these 8 genes, Hist1h2ad and 2ao have the same nucleotide sequence; however, the others have different nucleotide sequences. Quantitative RT-PCR analysis can be used to show the expression levels of different genes (for example [15]). Thus, in this study we designed the specific PCR primers for each histone H2a gene and studied the expression levels and patterns by qRT-PCR.

Results and discussion
Each product of the qRT-PCR gave a single band on the agarose gel, located in the expected position (Fig. 1). This result indicates that all histone H2a genes are expressed in Hepa 1–6 cells. The expression levels of 18 replication-dependent histone genes and H2afx increased along with cell cycle progression from the beginning (0 h) of S-phase to the middle (2–4 h) of S-phase, and then decreased from the middle to the end (6 h) of S-phase (Fig. 2). On the other hand, the expression level of the replication-independent gene H2afz lacked such a single peak during

![Figure 1](https://www.biomedcentral.com/1471-2164/6/108)

**Figure 1**
**RT-PCR products.** Lanes 1 and 19, DNA ladder marker; 2, Hist1h2aa transcript; 3, Hist1h2ab transcript; 4, Hist1h2ac transcript; 5, Hist1h2ad/1h2ao transcripts; 6, Hist1h2ae transcript; 7, Hist1h2af transcript; 8, Hist1h2ag transcript; 9, Hist1h2ah transcript; 10, Hist1h2al/1h2aj transcripts; 11, Hist1h2ak transcript; 12, Hist1h2an transcript; 13, Hist2h2ao1/2h2ao2 transcripts; 14, Hist2h2ao1/2h2ac transcripts; 15, Hist3h2a transcript; 16, H2afj transcript [9]; 17, H2afx transcript; 18, H2afz transcript.
S-phase (Fig. 2).

H2afz is regulated in a replication-independent manner, but H2afx is regulated in a replication-dependent manner. This pattern is consistent with the results of a previous report that indicated that H2afx gives rise to a cell-cycle-regulated mRNA ending in the stem-loop during S-phase, and a polyadenylated mRNA during G1-phase [10]. Therefore, H2afx is regulated in a replication-dependent manner (Fig. 2). On the other hand, H2afz lacks regulation of a polyadenylated mRNA. Interestingly, expression levels of H2afz decreased at the end (6 h) of S-phase, similar to those of replication-dependent genes (Fig. 2). This result suggests that the decrease at the end of S-phase is independent of the histone H2a mRNA structure.

We compared the sum of expression levels at 0, 1, 2, 3, 4, 5, and 6 h (S-phase) from each histone H2a gene (Fig. 3). Amino acid sequences from the proteins encoded by Hist1h2ab, 2ac, 2ad, 2ae, 2ag, 2ai, 2an, and 2ao were identical. However, among these 8 genes, the expression level of Hist1h2ae was 10 to 30 times that of Hist1h2ag (Fig. 3). Thus, the expression levels of the genes encoding the same structural protein were different.

Among the 13 genes in the Hist1 cluster, the expression level of Hist1h2ae was approximately 100 times that of Hist1h2aa (Fig. 3). In addition, the 4 genes in the Hist2 cluster had different expression levels. Thus, the expression level of Hist2h2a1/2a2 was approximately 10 times that of Hist2h2ab/2ac (Fig. 3). Therefore, the expression levels of genes belonging to the same gene cluster were different.

One possibility is that such different expression levels are caused by different promoters and different binding proteins bound to the promoters. For example, the promoters of Hist1h2ad, Hist1h2af, Hist1h2ag, and Hist1h2ah have the E2F binding motif (5'-TTTTCGCGCCC-3') between the CCAAT and TATA boxes [9]. Among these 4
replication-dependent genes, the expression level of Hist1h2ah was approximately 10 to 20 times that of Hist1h2ag (Fig. 3). In addition, compared among all 20 genes assessed in this paper, the expression levels of H2afz, Hist3h2a, Hist2h2aa1/2aa2, Hist1h2ae, and Hist1h2ai/aj were higher than that of Hist1h2ah, and those of Hist1h2ak and Hist1h2aa were lower than that of Hist1h2ag (Fig. 3). Thus, the relation between the E2F binding motif and the expression level is not clear. Unfortunately, we cannot determine here which structure of the promoters causes such different expression levels.

Next, we compared the histone H3 K9 acetylation levels in the promoter regions of Hist1h2aa (highest expression), Hist3h2a (highest expression among replication-dependent H2a genes), and Hist1h2aa (lowest expression). The ChIP-qPCR analysis showed that histone H3 K9 acetylation levels in the promoter regions of both H2afz and Hist3h2a were clearly higher than that in the promoter region of Hist1h2aa. The H3 K9 acetylation level in the promoter of Hist1h2aa was similar to that in the γ-satellite heterochromatin region (Table 1). This result indicates that the expression of histone H2a genes is related to the acetylation of histone H3 K9 in the promoter region.

## Conclusion
This study strongly suggests that histone H3 K9 acetylation plays a role in the expression of histone genes.

## Methods
### Cell cycle synchronization
The cell cycle of mouse Hepa 1–6 cells was synchronized at the end of G1-phase by the addition of thymidine-hydroxyurea. The cell cycle arrest was released by washing out the thymidine-hydroxyurea, then the cells were harvested at intervals of 1 h from 0 to 13 h.

### RNA extraction
Total RNA was extracted by using the RNeasy mini kit (Qiagen) according to the instructions in the manual for
the cell line. After that, each sample was treated with DNase I.

cDNA synthesis
RNA (approximately 0.5 µg) and random hexamer primers were heated to 70°C for 10 min, followed by cooling on ice for 5 min. The cDNA was synthesized in Superscript III First Strand buffer (Invitrogen) according to the manual. The reverse transcriptase was inactivated by a 15-min incubation at 70°C.

Quantitative PCR
The primers used in this analysis are shown in Table 2. Quantification of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA (primers 5’-TGTGTCCGTCGTGGATCTGA-3’ and 5’-CCTGCTTCACCACCTTCTTG-3’; product size 76 bp) was used as a control for data normalization. PCR amplification was performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The PCR conditions were an initial step of 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. The SYBR premix Ex Taq (Takara) was used according to the manual. Each amplification curve was checked [16]. Expression was assessed by evaluating threshold cycle (CT) values. The relative amount of expressed RNA was calculated by using Livak and Schmittgen’s method [17]. The qRT-PCR analyses were performed twice. In each analysis, we adjusted the $H2afz$ expression level to 1 at 0 h.

Chromatin immunoprecipitation
A total of $2 \times 10^7$ cells were cross-linked with 1% formaldehyde for 10 min at room temperature. First, genomic DNA was cut by micrococcal nuclease. Then it was cut by sonication. The precleared extract was divided into 2 equal portions. One was used for control lacking antibody, and the other was incubated with acetylated histone H3 K9 antibody (Upstate Biotechnology). Following immunoprecipitation, beads were washed in low salt, then high salt, then LiCl, then TE buffers. The qPCR analyses were performed two times. Primers used in quantitative PCR were the $Hist1h2aa$ promoter (5’-TTATAGCGTGGACATT-3’ and 5’-CACAGCGTGGACATT-3’), the $Hist3h2a$ promoter (5’-CCGCGTTCTTTTCTGGAT-3’ and 5’-AATTCGTAAGCGCCAGC-3’), and the $H2afz$ promoter (5’-GGCCAATCATCGCTCG-3’ and 5’-TCGGGACGCGTCCTTGA-3’). We used γ-satellite as a constitutive heterochromatin. The γ-satellite PCR primers have been reported [18].

Authors’ contributions
HN designed this study and carried out the molecular biological studies. TS and HO carried out the ChIP experiment and qPCR. YT carried out synchronization of cells. YH helped design the study.

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