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Global Histone H4 Acetylation of IGF1 and GH Genes in Lungs of Somatic Cell Cloned Calves

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ABSTRACT : Histone acetylation modification is one key mechanism in the regulation of gene activation. In this study, we investigated the global levels of histone H4 acetylation of insulin like growth factor I (IGF1) and growth hormone (GH) genes in the lungs of two somatic cell cloned calves. Data showed the levels of histone H4 acetylation of IGF1 and GH genes vary widely within different gene regions, and, in almost all regions of the two genes, acetylation levels are lower in the aberrant clone than in the normal clone. Thus we suggest that inefficient epigenetic reprogramming in the clone may affect the balance between acetylation and deacetylation, which will affect normal growth and development. These findings will also have implications for improvement of cloning success rates. (**Key Words :** Histone H4 Acetylation, Somatic Cloned Calves, IGF1, GH, Lung)

INTRODUCTION

Although some mammalian species have been successfully cloned by somatic cell nuclear transfer, only a small proportion of embryos reconstructed using adult or fetal somatic cells developed to become live young. The low overall success rate is the cumulative result of inefficiencies at each stage of the process (Wilmut et al., 2002). Recent successes in mammalian cloning with differentiated adult nuclei strongly indicate that the oocyte cytoplasm contains unidentified important reprogramming activities with the capacity to erase the previous memory of cell differentiation (Wade and Kikyo, 2002). Many experiments have demonstrated epigenetic reprogramming in cloned animals, including chromatin structure, DNA methylation, imprinting, telomere length adjustment, and X chromosome inactivation. Inefficient epigenetic reprogramming can result in inappropriate gene expression. Embryos may die at various stages of pre- or postnatal development depending on a certain threshold for faulty expression of the particular genes affected in a given clone or because of the random dysregulation of a key gene(s) crucial for a specific developmental stage (Rideout et al., 2001).

Histone acetylation is one key epigenetic modification to regulatie gene activation. The amino termini of histones extend from the nucleosomal core and are modified by histones acetyltransferases (HATs) and histone deacetylases (HDACs) during the cell cycle. As acetylation neutralizes the positively charged lysine residues of the histone N termini, decreasing their affinity for DNA, this might allow the termini to be displaced from the nucleosome, causing the nucleosomes to unfold and increasing access to transcription factors (Grunstein, 1997). Generally, actively transcribed chromatin regions have been associated with hyperacetylation and histone acetyltransferase recruitment. The balance between acetylation and deacetylation is an important factor in regulating gene expression and is thus linked to the control of cell fate. As a consequence, hyperacetylation of normally silenced regions or deacetylation of normally actively transcribed region can lead to various disorders, including developmental and proliferative diseases (Timmermann et al., 2001). Potentially active euchromatin can be modified at all the H4 acetylatable lysines (K5, K8, K12 and K16), whereas H4 in heterochromatin is hypoacetylated (Clarke et al., 1993; O'Neill and Turner, 1995). Some promoters are strongly acetylated or deacetylated in promoter-specific manner, however, the surrounding regions containing these sites are

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Table 1. Primers for IGF1 gene in real-time quantitative PCR

No.	Forward primers (5'→3')	Reverse primers (5'→3')	Primer location	PCR product (bp)
P1	TGAAACGGCATCACTTCTCAT	GGGTGCCCTCAAAGGAAA	317/432	133
P2	CCATGGACTAAAAAGACCCAG	GCCCAGGTTAGACATCCCACT	471/630	180
P3	GGCTTTTGCAATCTTATTTCA	ATATTTGCCTTTGCCATTGAG	6,48/751	124
P4	AAGCAGCAGAGTAGAAGGAAG	GGGCAGGCAGCTTTGTAATTG	1,062/1,185	144
P5	GCGCTGTCTTCCATTCTAGTT	ATACACCTTACCCGTATGAAA	1,271/1,419	169
P6	CCCGGTAGAAAGTTAATCAG	GGGCATGGTGACAAATAACATCA	1,463/1,586	146
P7	CCCCCAGCTGTTTCCTGTCTACA	TATTCCATTGCGCAGGCTCTATC	1,668/1,780	135
P8	AGGACGGCTACAATAGGCAC	TTGGACACCCAGGCAGGTATGCT	2,083/2,209	149
P9	TGCCTGGGTGTCCAAATGTAACT	CAGCAGGTCAGGGTGGGTATT	2,217/2,318	122
P10	CACATCCTCCTCGCATCTC	CATTCAGTTCTTCGCACACTC	2,560/2,749	210
P11	CTCCCTCTCGCTGCTCTGTGG	GCATTAAGGTGAGGAATCTCG	2,712/2,801	110
P12	AGGAGGCTGGAGATGTACTGC	CTGCTGCTGCTAAGTTGCTACAG	2,908/3,006	184
P13	TGTCACTTTTTCTCGCTTATT	CGGTGGCATGTCATTCTTCAC	3,225/3,328	124
P14	TGACCCTGGAGTTGGTAGATT	TCTATACAACACCCATGCATT	3,513/3,612	120

Table 2. Primers for GH gene in real-time quantitative PCR

No.	Forward primers (5'→3')	Reverse primers (5'→3')	Primer location	PCR product (bp)
P1	GGAACGGGAACAGGATGAGT	CTGGGATCCTGGAATTGGTCC	26/170	165
P2	CCTCCTGGTCTCTCCCTAGG	GAGCACAGCGTTGGCAAAC	566/679	132
P3	GCTCCCGAGGGATGCGTC	CCGTTTCTGCTCCCCTAACC	750/865	135
P4	GATACTCCATCCAGAACACCC	TTGCCACTCACTGATTTCTGC	1,001/1,080	100
P5	GGATGATGGTGGGCGGTGGTG	TGCGAAGCAGCTCCAAGTCCT	1,217/1,317	121
P6	ACTTGGAGCTGCTTCGCATCT	AAGGGACCCACAACGCCATC	1,320/1,486	186
P7	CTTAGCCAGGAGAATGCACG	ATTTTCCACCCTCCCCTAC	1,538/1,670	151
P8	TGGGCAGATCCTCAAGCAGAC	GTCTCCGTCTTATGCAGGTCC	1,781/1,880	120

also acetylated and deacetylated (Vogelauer et al., 2000).

The IGFs are synthesized locally and have potential paracrine and autocrine actions in tissues and can affect a wide variety of cell-specific functions (Jones and Clemmons 1995), even in relation to mammary blood flow and milk yield in cattle (Chaiyabutr et al., 2004). IGFs are important mitogens involved in lung growth and development (Schuller et al., 1995) and IGF-1 mRNA remained relatively constant in fetal and adult lungs (Moats-Staats et al., 1995; Schuller et al., 1995). GH is secreted in the pituitary gland and is a member of a large class of evolutionarily related protein hormones. GH regulates a wide range of biological processes (Kopchick and Andry, 2000), but is not expressed in lung.

In this study, we analyzed the histone H4 acetylation in distinct domains of IGF1 and GH genes in lungs of two somatic cell cloned calves.

MATERIALS AND METHODS

Animal

The somatic cell cloned Holstein calves C1 and C2 were produced by nuclear transfer (Gong et al., 2004) in our lab, and the donor nuclei were obtained from adult fibroblast cells and cumulus cells, respectively. C1 died in gestation (252 days) with many abnormalities, especially in lung (atelectasis, thicken of alveolar wall). C2 was healthy and slaughtered on day 4 after birth. Particularly, C2 had no any respiratory and pathologic defect. The clones were dissected immediately after death and major internal organs were collected and frozen in liquid nitrogen, then refrigerated at -70°C.

Acetyl-histone H4 immunoprecipitation (ChIP) assay

Approximately 1 mg lung tissue (≈ 1×10^6 cells) was placed in a 2 ml microcentrifuge tube, and 1 ml 1% formaldehyde and homogenize tissue was added. After a 30 min incubation on ice for cross linking histone to DNA, an Acetyl-Histone H4 Immunoprecipitation (ChIP) Assay Kit was used to prepare immunoprecipitated DNA (Upstate Biotechnology, Lake Placid, NY, USA). In these processes, the protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin and pepstatin A) (Roche, Mannheim, Germany) are always used. DNA is recovered and purified by the Wizard DNA Clean-up System (Promega, Madison, WI, USA). Increased PCR amplification of a sequence reflects the increased acetylation at that chromosomal site. The process was repeated 3 times for each sample and then samples were combined.

Primer design

We chose 14 pairs of primers (Table 1) for the IGF1 gene (GenBank No.AF210383) and 8 pairs of primers (Table 2) for the GH gene (GenBank No.J00008) in real-time PCR analysis. The PCR annealing temperatures of primer pairs were determined using Mastercycler Gradient

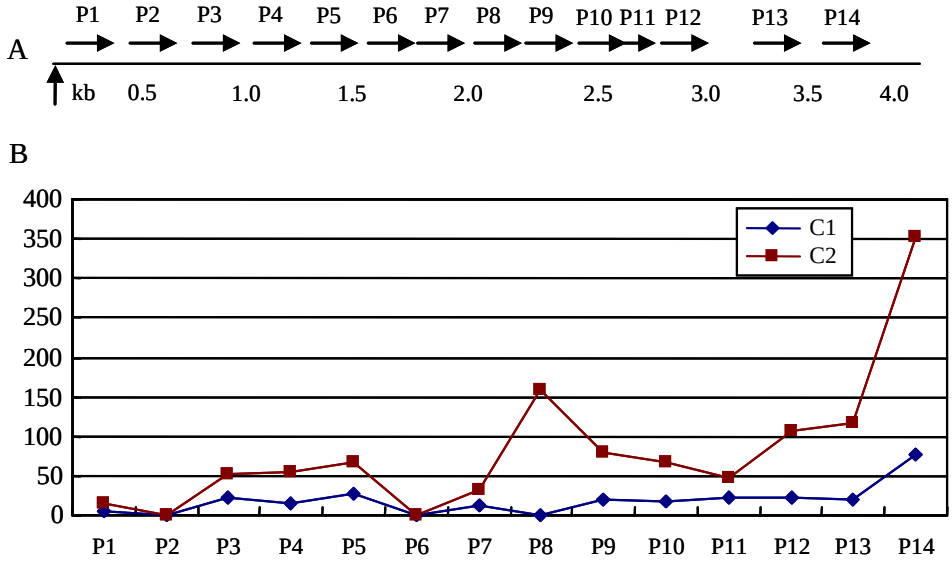

Figure 1. A: Schematic of the 4 kb region encompassing the bovine IGF1 gene. Vertical arrow indicates the transcriptional initiation site. Horizontal arrows with numbers P1 to P14 indicate position of the Real-time PCR fragment relative to the genomic region. B: Global levels of histone H4 acetylation of IGF1 gene in cloned calves C1 and C2. Vertical values indicate the relative acetylation levels.

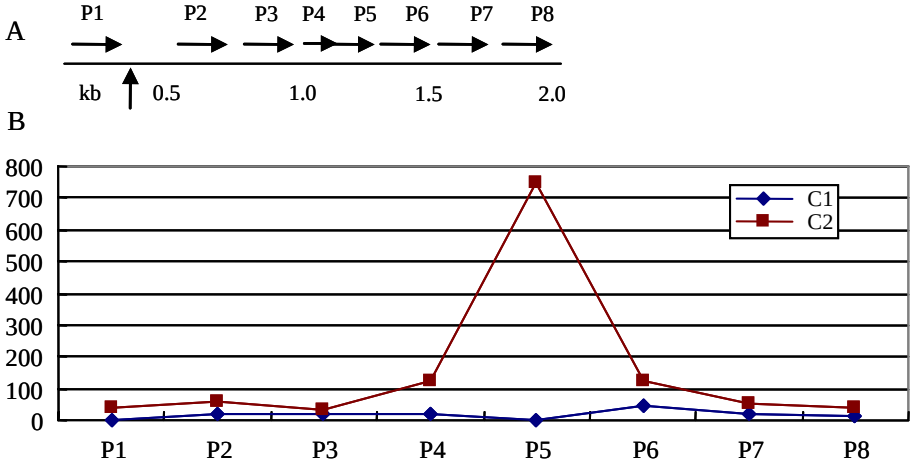

Figure 2. A: Schematic of the 2 kb region encompassing bovine GH gene. Vertical arrow indicates transcriptional initiation site. Horizontal arrows with numbers P1 to P8 indicate position of the real-time PCR fragment relative to the genomic region. B: Global levels of histone H4 acetylation of GH gene in cloned calves C1 and C2. Vertical values indicate the relative acetylation levels.

(Eppendorf, Hamburg, Germany).

Real-time quantitative PCR (Q-PCR)

Real-time PCR was carried out using the SYBR PCR Reagents Kit and the ABI Prims 7900HT Sequence Detection System (Applied Biosystems, Foster, USA). The 20 μl reaction mixture contained 1×SYBR Green I PCR Mastermix, 0.1 μM of each primer, and 1 μl of template. The PCR conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 61°C/62°C (respectively for IGF1 and GH) for 30 s, 72°C for 1 min. Melting curves were generated after amplification. A standard curve was created by a 10-fold dilution over a range spanning the sample concentrations with product of general PCR amplifications (product of IGF1-3 primer pairs for all primer pairs in IGF1 analysis, and product of GH-2 primer pairs for all primer pairs in GH analysis). The correlation was at least 0.998. Negative control was done without template in the PCR system. Each sample was tested 4 times, and the standard curve was repeated 3 times. Samples with C_T values >35 are treated as negative. In real-time PCR analysis, a house-keeping gene β-actin (Forward primers: 5'- GCTCGCCAT CAGTTACAAG-3'; reverse primers: 5'-AATGCCTTGG CTCCCTAGATG-3'; annealing temperature 56°C) was used to quantitate the amount of DNA present in different samples from ChIP by real-time PCR, and C2 DNA template was used to quantity the differences within primer pairs.

RESULTS

The levels of histone H4 acetylation in distinct regions of IGF1 gene vary widely in both samples (Figure 1) and acetylation levels of distinct regions is always higher in C2 than in C1, except the two regions P2 and P6 in which acetylation did not been examined. We also observe P14 has the highest levels and P1 has the lowest levels in each sample. In C1, the highest acetylation levels in P14 is 14.8-fold higher than the lowest levels in P1; whereas, in C2, the highest acetylation levels in P14 is 22.3-fold higher than the lowest no-zero levels in P1. In addition, in most cases, the distinct regions of the two samples change in a similar manner.

The levels of histone H4 acetylation in distinct regions of GH gene also vary widely in both samples (Figure 2) and the distinct regions of the two samples change in a similar manner with the exception of the P5 region. In the P5 region, we did not examine acetylation in C1, however, we examined the highest levels in C2 (24.9-fold higher than the lowest no-zero levels).

DISCUSSION

Global acetylation and deacetylation of a chromosomal region analysis indicated the distinct gene regions have different acetylation levels (Vogelauer et al., 2000). Liang et al. have shown 6- to 122-fold variation at the start sites relative to the downstream region of some human genes (Liang et al., 2004). In this study, the histone H4 acetylation of the two genes was examined in the 5' flanking region, promoter and coding region. The levels of acetylation are very variable in distinct regions and the variable acetylation levels indicate the transcribing activation of distinct chromatin regions. In particular, although the GH gene is expressed in the pituitary gland and should not been expressed in the lung, we examined acetylation in most regions. This indicates the silent chromatin regions also have global acetylation modification and maintain some levels. Vogelauer et al. (2000) have indicated that the global modification may have two important functions. First, the balance of acetylation/deacetylation prevents full acetylation, thereby creating a default underacetylated state. This may decrease basal transcription of many genes by reducing both initiation and elongation of transcription. Secondly, the rapid turnover of acetyl groups at most nucleosomes may allow chromatin to revert to the initial default acetylation state when targeting is removed. This would prevent the irreversible hyperacetylation or deacetylation of promoters and other regions.

For successful development of the reconstructed embryo, the transferred nucleus must be reprogrammed to establish the temporal and spatial gene expression patters associated with normal development. The inefficient epigenetic reprogramming could result in the inappropriate gene expression (Wade and Kikyo, 2002). The increasing levels of histones acetylation may reduce the interaction between histones and DNA and thereby improve access of transcription factors to their cognate sequences on nucleosomal DNA (Turner, 1998). In most case, acetylation levels of each region in C1 are always lower than that in C2. C2 was very healthy, whereas C1 was abnormal. The decreased acetylation levels might have some contributions to the C1 abnormalities. This is also has implications for improvement in cloning, such as some deacetylation inhibitors or acetylation activators would be used to increased acetylation levels in the reconstructed embryo, so as to improve the success rate of cloning. Some studies have displayed that donor cells with Trichostatin A (Enright et al., 2003) and sodium butyrate (Shi et al., 2003) (histone deacetylation inhibitors) treatment increased blastocyst development of cloned embryos.

Taken together, in this investigation, although we just analyzed two samples, the results are also useful to get some important and interesting conclusions. We examined histone H4 acetylation in both IGF1 and GH genes, and the actylation levels vary widely within different gene regions. And more, decreased acetylation levels might contribute to the aberrant growth and development in somatic cell cloned calves. In the future experiments, we should examine more samples including normal calf lung samples.

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