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Hirotaka Yoshioka · Yasuaki Shirayoshi
Mitsuo Oshimura

A novel in vitro system for analyzing parental allele-specific histone acetylation in genomic imprinting

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Abstract One of the obstacles in studying human genomic imprinting is distinguishing the parental origin of alleles in diploid cells. To solve this problem, we have constructed a library of mouse A9 hybrids in which individual clones contain a single human chromosome of known parental origin. Here we extend this in vitro system to the analysis of the role of histone acetylation in the allelic expression of human imprinted genes. The levels of histone H4 acetylation of the imprinted human *LIT1*, *H19*, and *SNRPN* genes were examined by a chromatin immunoprecipitation (ChIP) assay in mouse A9 hybrids with a single human chromosome of known parental origin. We demonstrated that H4 histones associated with the actively expressed alleles of imprinted *LIT1*, *H19*, and *SNRPN* genes were highly acetylated, whereas they were hypoacetylated in the silent alleles. Furthermore, treatment of A9 hybrids with trichostatin A (TSA), an inhibitor of histone deacetylase, resulted in transcriptional reactivation of the silent alleles for *LIT1* and *SNRPN*, suggesting that histone deacetylation is one of the key regulatory mechanisms in genomic imprinting. These results indicate that our monochromosomal hybrid system is a new technology for analyzing histone modifications between parental alleles in human imprinted genes.

Key words Genomic imprinting · Monochromosomal hybrid cells · DNA methylation · Histone acetylation · Trichostatin A · Chromatin immunoprecipitation assay

H. Yoshioka · Y. Shirayoshi · M. Oshimura (✉)
Core Research for Evolutional Science and Technology (CREST) project, Department of Molecular and Cell Genetics, School of Life Sciences, Faculty of Medicine, Tottori University, 86 Nishimachi, Yonago, Tottori 683-8503, Japan
Tel. +81-859-34-8260; Fax +81-859-34-8134
e-mail: oshimura@grape.med.tottori-u.ac.jp

Introduction

Genomic imprinting is thought to result from differential epigenetic modifications of alleles of different parental origin that lead to parent-of-origin-specific expression (Reik and Walter 2001). Causative mechanisms of genomic imprinting such as cytosine methylation in CpG dinucleotides (Li et al. 1992), chromatin structure (Feil and Kelsey 1997), and replication timing (Simon et al. 1999) have become clear in studies of many human and mouse imprinted genes. The in vivo mutational studies of related DNA methyltransferase genes revealed that cytosine methylation in CpG dinucleotides has a great influence on the maintenance of parental allele-specific expression of imprinted genes and on mammalian development (Li et al. 1992; Okano et al. 1999). Recently, it has been demonstrated that the methyl cytosine binding protein MeCP2 binds the corepressor of mSin3A, which constitutes the core multiprotein complex including histone deacetylases HDAC1 and HDAC2 (Jones et al. 1998; Nan et al. 1998), and that DNA methyltransferase Dnmt1 is itself associated with histone deacetylase activity (Fuks et al. 2000). Furthermore, epigenetic gene regulation is accomplished through the modulation of chromatin packaging that results in several forms of posttranscriptional modification of histones (Wolffe and Matzke 1999). Thus, the process of DNA methylation may generate an altered chromatin state via histone deacetylase activity for allele-specific epigenetic modification in genomic imprinting.

The presence of both paternal and maternal genomes in diploid cells is a significant barrier to the analysis of the mechanisms of genomic imprinting, including histone modification. Genetic polymorphisms in over 160 bp DNA wrapped around an octamer of the four core histones are required for analyzing the parent-of-origin-specific histone modifications of each nucleosome in diploid cells. However, the analysis of critical nucleosomes for epigenetic regulation could be hampered by rare incidences of nucleotide sequence variations. Indeed, the analysis of allele-specific histone acetylation of imprinted genes has been limited to

regions of the genome exhibiting polymorphism, as shown in Pedone et al. (1999). We have constructed a library of mouse A9 hybrids containing single human paternal or maternal chromosomes via microcell-mediated chromosome transfer (Kugoh et al. 1999). The parent-of-origin-specific expression and DNA methylation of human imprinted genes, such as *H19*, *SNRPN*, and *IPW*, were maintained faithfully (Meguro et al. 1997; Mitsuya et al. 1998) as was the replication timing of *H19* (unpublished data) in these A9 hybrids.

In this article, we have applied our monochromosomal hybrid system to the analysis of the relative levels of histone acetylation in human imprinted genes. To gain insight into the potential significance of histone acetylation in the regulation of genomic imprinting, the relative levels of allele-specific histone H4 acetylation were investigated by using a chromatin immunoprecipitation (ChIP) assay with antibodies against acetylated histone H4 (Crane-Robinson et al. 1999). In addition, the expression profiles of several imprinted genes were compared in trichostatin A (TSA)-treated and untreated A9 hybrids. We demonstrate here that differential acetylation of histone H4 between parental alleles is associated with the epigenetic regulation of some imprinted genes. Thus, this monochromosomal hybrid system enables efficient analysis for demonstrating differences in histone modification patterns between alleles of maternal and paternal origin.

Materials and methods

Cell culture and TSA treatment

Mouse A9 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA), plus 10% bovine calf serum at 37°C, 5% CO_2. Mouse A9 hybrids containing a single human chromosome, either 11 or 15, of known parental origin (Kugoh et al. 1999), were cultured under the same conditions as A9 cells with 3 μg/ml blasticidin S hydrochloride (Invitrogen, Groningen, The Netherlands). Normal human fibroblasts were obtained with standard punch biopsies from a normal adult and cultured in DMEM supplemented with 10% fetal bovine serum at 37°C, 5% CO_2.

For TSA treatment, human fibroblasts, mouse A9 cells, and mouse A9 hybrids were plated at a density of 5×10^6 cells/dish with 100 ng/ml of TSA (Wako Pure Chemicals, Osaka, Japan). After 24 hours, all the cells were harvested for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

Chromatin immunoprecipitation assay

For chromatin preparation, 10^8 cells were washed with ice-cold phosphate-buffered solution (PBS) in the presence of protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml pepstatin] (Roche Diagnostics, Indianapolis, IN, USA), and resuspended in N1 buffer [15 mM Tris-HCl pH 7.6, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.1 mM ethyleneglycoltetraacetic acid (EGTA) pH 7.8, 0.3 M sucrose, 0.5 mM dithiothreitol (DTT), 0.1 mM PMSF, 5 mM sodium butyrate]. After permeabilization with addition of Nonidet P-40, nuclei were recovered by ultracentrifugation through an N2 buffer cushion (15 mM Tris-HCl pH 7.6, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA pH 7.8, 1.5 M sucrose, 0.5 mM DTT, 0.1 mM PMSF, 5 mM sodium butyrate). The nuclear pellets were then resuspended in micrococcal nuclease (MNase) buffer (20 mM Tris-HCl pH 8.0, 5 mM NaCl, 2.5 mM $CaCl_2$) and digested with 100 units of MNase (Takara, Kyoto, Japan) at 37°C for 10 min to isolate the mononucleosomes and the smaller oligonucleosomes. The MNase reaction was stopped by adding a final concentration of 5 mM ethylenediaminetetraacetate pH 8.0. Mononucleosome and the smaller oligonucleosome preparations were subjected to immunoprecipitation with antibodies against acetyl histone H4 using an Acetyl-Histone H4 Immunoprecipitation Assay Kit (Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer's instructions with minor modifications.

Multiplex PCR analysis of chromatin immunoprecipitated DNA

Immunoprecipitated DNA was analyzed using the following primers and PCR conditions. The human-specific PCR primers for imprinted genes used were LIT1-F: 5′-GTTG CTGAGGTGAGCTGTGT-3′ and LIT1-R: 5′-TGTCCAT AAGGTGCAGATGG-3′ for *LIT1*; H19-1F: 5′-TTCTGG GAATAGGACGCTCA-3′ and H19-1R: 5′-CCAGGCCA TGACACTGAAG-3′, H19-2F: 5′-CGGTCTTCAGACA GGAAAGTG-3′ and H19-2R: 5′-ATCCAGTTGACCGA GCTTGT-3′ for *H19*; and SNT5.9-F: 5′-CAGGCTTCGCA CACATCC-3′ and SNT5.9-R: 5′-CAGGTCATTCCGG TGAGG-3′ for *SNRPN*. The mouse *G6pd* promoter was amplified by multiplex PCR as an internal control in the same reaction tube with each imprinted gene using the PCR primers G6pd-F: 5′-ATTTTCAAGGCACCGCATC-3′ and G6pd-R: 5′-CTAGTTTGGCTTCGGAGCTG-3′. The PCR conditions were 31 cycles of 94, 62, and 72°C for 30, 30, and 30 s, respectively, for *LIT1* and *SNRPN*, and 31 cycles of 94, 60, and 72°C for 30, 30, and 30 s, respectively, for *H19*. PCR products were quantified with the fluorescent image-analyzing system FMBIOII Multi-View (Takara) to normalize for *G6pd* products and electrophoresed on 12% polyacrylamide gel, followed by ethidium bromide staining.

Expression analysis by RT-PCR

Total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) and treated with DNase I (Roche Diagnostics) to remove any DNA contamination. First-strand cDNA synthesis was carried out with (+) or without (−) M-MLV reverse transcriptase (Gibco BRL,

Rockville, MD, USA) using an oligo $(dT)_{15}$ primer (Roche Diagnostics).

The expression of human imprinted genes was detected by using the human-specific primers Lit102: 5′-TCTGGT TCATGTCACTCTGTGGAGCAG-3′ and Lit202: 5′-CTCCCAAAAGCAGAGTTTTGGCAATAT-3′ for *LIT1* (Mitsuya et al. 1999); H1: 5′-TACAACCACTGCA CTACCTG-3′ and H3: 5′-TGGAATGCTTGAAGGCT GCT-3′ for *H19* (Rainier et al. 1993); and S4: 5′-CTACTC TTTGAAGCTTCTGCC-3′ and AS4: 5′-TGAAGATTCG GCCATCTTGC-3′ for *SNRPN*. To detect the expression of *LIT1*, we used a step-down PCR protocol. Nine rounds of PCR were carried out as follows, with the annealing temperature being reduced by 2°C every three cycles: 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s. Thirty-five subsequent rounds of PCR were carried out as follows: 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s. The other PCR conditions were 35 cycles of 94°C for 45 s, 62°C for 30 s, and 72°C for 90 s for *SNRPN* and 34 cycles of 94°C for 1 min, 62°C for 3 min, and 72°C for 5 min for *H19*.

Results

Histone H4 acetylation of imprinted genes

To examine the role of histone acetylation in genomic imprinting, we used a series of mouse A9 hybrid cells containing a single human paternal or maternal chromosome as a source for chromatin preparation. The imprinted human genes, *LIT1* and *H19* on chromosome 11 and *SNRPN* on chromosome 15, were selected for analysis of allelic histone H4 acetylation using the ChIP assay (Crane-Robinson et al. 1999). In the A9 hybrids, the epigenetic status of the transferred human chromosome is maintained faithfully; for example, the expression pattern is dependent on the parental origin and the DNA methylation status (Meguro et al. 1997; Mitsuya et al. 1998, 1999; Kugoh et al. 1999).

Chromatin was prepared from A9 hybrids and immunoprecipitated with antibodies raised against acetylated histone H4. In immunoprecipitated nucleosomes (bound fraction), DNA fragments containing the sequences described below were amplified by multiplex PCR with primers for a positive control gene, in this case *G6pd*, which allowed for a semi-quantitative assay of association of a particular DNA sequence with acetylated histones. Amplification of DNA was compared between A9 hybrids containing either human paternal chromosome (paternal A9 hybrids) or the corresponding maternal chromosome (maternal A9 hybrids).

We first analyzed histone H4 acetylation in a region spanning the maternally methylated CpG island of *LIT1*, which overlapped with two clusters of short direct repeat sequences (Fig. 1A). PCR primers for *LIT1* were chosen from a region between the two direct repeat clusters because of the difficulty with generating primers to the repeat regions. Equal amounts of DNA from input, unbound, and bound nucleosomes, which were based on *G6pd* amplification, were amplified and compared between the paternal and maternal A9 hybrids. The input and unbound fractions gave bands of approximately equal intensity, irrespective of their parental origin (data not shown). However, only the *LIT1* alleles from two independent paternal A9 hybrids, A9(11P)-1 and A9(11P)-2, were amplified in the bound fraction (Fig. 1B). Virtually, no amplification was observed for the *LIT1* alleles from the maternal A9 hybrids, A9(11M)-1 and A9(11M)-2. Therefore, in the bound fraction a marked difference exists in the relative amplification level of *LIT1* alleles in the two parental A9 hybrids.

To investigate the histone H4 acetylation status of the *H19* gene, we first analyzed the paternally methylated CpG island in the 5′ *cis*-regulatory element (Fig. 1A, H19-1), which has potential insulator activity (Bell and Felsenfeld 2000; Hark et al. 2000). Using the same amounts of immunoprecipitated nucleosomes, the *H19* alleles in maternal hybrids were amplified strongly (Fig. 1C, H19-1). On the other hand, the paternal *H19* alleles were amplified poorly compared with the maternal alleles. Although less evident, similar results were obtained by PCR amplification using different primers for *H19* alleles corresponding to the CpG island of the promoter region (Fig. 1C, H19-2) (Dugimont et al. 1998). Therefore, the maternal *H19* allele, the transcriptionally active allele, is suggested to be more acetylated than the paternal allele.

Because the *LIT1* and *H19* genes are located on chromosome 11p15.5, we next analyzed the chromatin acetylation status of the *SNRPN* gene using A9 hybrids containing human chromosome 15. The maternally methylated CpG island surrounding exon 1 of the *SNRPN* gene is indispensable for the establishment of the paternal imprint in the male germ line (Fig. 2A) (Bielinska et al. 2000). Therefore, we chose PCR primers for *SNRPN* alleles from this region. As with the *LIT1* gene, only the active paternal alleles were heavily immunoprecipitated by anti-acetylated histone H4 antibodies, indicating that a clear difference exists in the acetylation status between the paternal and maternal A9 hybrids (Fig. 2B).

Effect of inhibition of histone deacetylase

Inhibition of histone deacetylase with TSA increases the acetylation level of histones (Yoshida et al. 1990) and activates gene transcription in some cases (Kouzarides 1999). To test the relevance of histone acetylation to the relative transcriptional activity of the paternal and maternal alleles of imprinted genes, we analyzed the effects of TSA on the expression status of *LIT1*, *H19*, and *SNRPN* in A9 hybrids. RT-PCR analysis showed that untreated A9 hybrids maintained the imprinting of the *LIT1*, *SNRPN*, and *H19* genes, which was exclusive paternal, paternal, and maternal expression, respectively (Fig. 3). Treatment of the A9 hybrids with 100 ng/ml of TSA altered the expression of the *LIT1* and *SNRPN* genes, since TSA reactivated the normally silent *LIT1* and *SNRPN* genes with a maternal origin (Fig. 3A and 3B). These results suggest that histone acetylation plays a crucial role in maintaining allele-specific expression

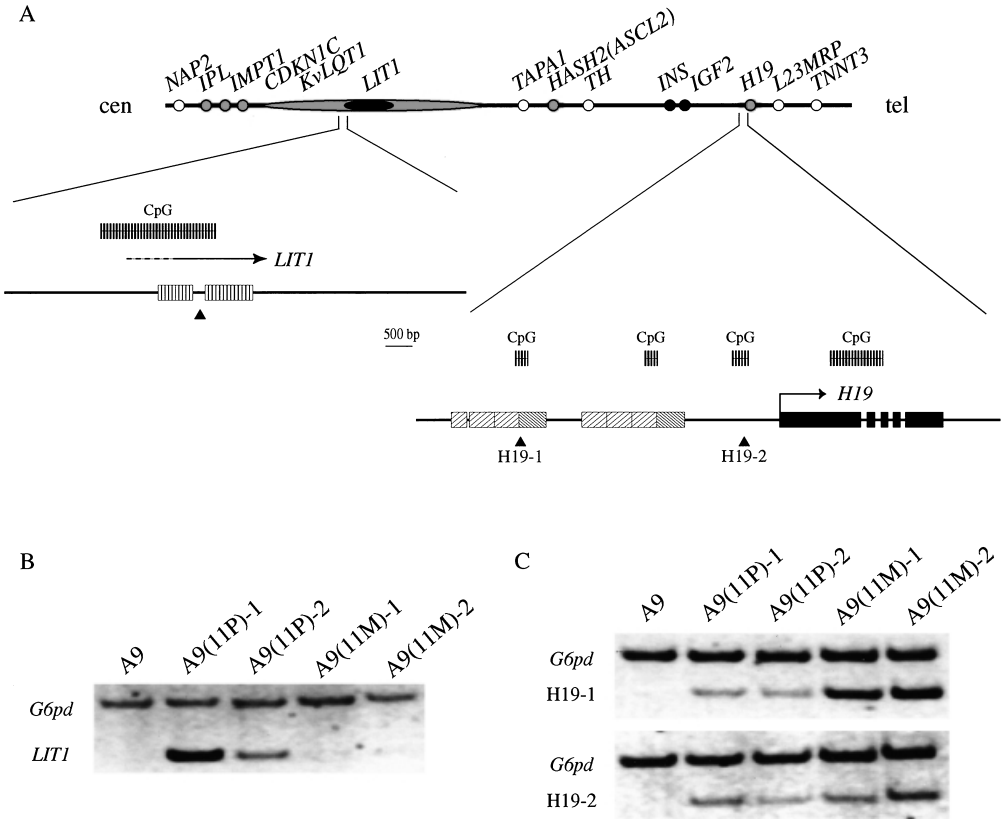

Fig. 1. A–C. Chromatin immunoprecipitation assay of the imprinted *LIT1* and *H19* genes on human chromosome 11p15.5. **A** Overview of the imprinted domain on human chromosome 11p15.5. *Black*, *gray*, and *white circles* indicate paternally expressed imprinted genes, maternally expressed imprinted genes, and nonimprinted genes, respectively. In the *lower left panel*, the structure of the differential methylated *CpG island* of *LIT1* is shown. This region is characterized by two clusters of short direct repeat sequences (*striped boxes*). We analyzed the relative levels of histone H4 acetylation in a region between these two direct repeat clusters (*arrowhead*). The *LIT1* transcriptional start site has not been determined clearly, as indicated by the *dotted line* and *arrow*. In the *lower right panel*, the structure of the upstream differential methylated *CpG islands* of *H19* is shown. Two types of 400 bp repeat sequences (*hatched boxes*) are indicated. Two regions indicated with *arrowheads*, *H19-1* and *H19-2*, were analyzed by a chromatin immuno precipitation assay. The *black boxes* and the *arrow* indicate the exons and transcriptional start site, respectively. **B, C** Multiplex polymerase chain reaction (PCR) analysis of DNA in chromatin immunoprecipitated with anti-acetyl-H4 antibodies from mouse A9 recipient cells and mouse A9 hybrids with paternal chromosome 11 *A9(11P)* clones, and with maternal chromosome 11 *A9(11M)* clones. DNA from antibody-bound chromatin fractions was amplified by multiplex PCR using primer pairs for *G6pd* (*top band*) and each imprinted gene (*bottom band*), *LIT1* (**B**) and *H19* (**C**)

of *LIT1* and *SNRPN*. In contrast, the same TSA treatment in mouse A9 hybrids did not alter the exclusive maternal expression status of *H19*, indicating that restoring acetylation of histones was insufficient to reactivate *H19* (Fig. 3C). Thus, there seems to be at least two types of regulatory systems leading to the silencing of one allele of imprinted genes.

Discussion

In this study, we introduced a new method for studying the histone modifications in imprinted genes using a monochromosomal hybrid system (Kugoh et al. 1999). This method is based on the fact that the epigenetic status of human imprinted genes is maintained faithfully in A9 hybrids containing a single human chromosome. Since polymorphisms are not required to distinguish parental alleles in our system, the monochromosomal A9 hybrids enable us to examine the chromatin structure throughout the genomic regions of the imprinted genes.

Using A9 hybrids with human chromosome 11, we found that the silent and hypermethylated *LIT1* and *H19* alleles were hypoacetylated, although *LIT1* and *H19* belong to different imprinted subdomains of human chromosome 11p15.5 (Lee et al. 1999; Mitsuya et al. 1999; Horike et al. 2000). Conversely, histones of the expressed and undermethylated *LIT1* and *H19* alleles were hyperacetylated. In the case of *H19*, the status of histone acetylation was likely to be maintained throughout the 5′ *cis*-regulatory element because the CpG island of the *H19* promoter showed the same allelic histone acetylation as the upstream CpG island. The same relationship between acetylation and methylation was observed in imprinted *SNRPN* alleles using different A9 hybrids containing human chromosome 15. This finding confirmed recent reports that the two alleles of *SNRPN* displayed different levels of histone acetylation (Saitoh and Wada 2000; Fulmer-Smentek and Francke 2001), validating the value of human

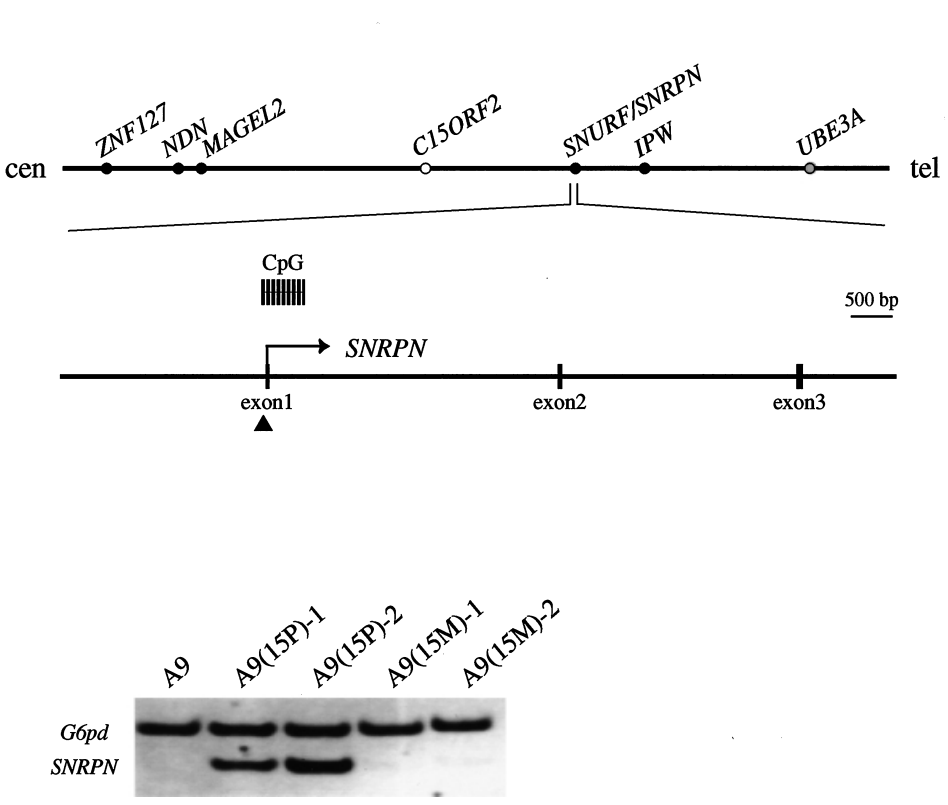

Fig. 2. A, B. Chromatin immunoprecipitation assay of the imprinted *SNRPN* gene on human chromosome 15q11–q13. **A** Overview of the imprinted domain on human chromosome 15q11–q13 and the structure of the upstream region of the *SNRPN* locus. *Black boxes* represent exons and the *arrow* indicates transcription from *exon 1*. **B** Multiplex PCR analysis of DNA in chromatin immunoprecipitated with anti-acetyl-H4 antibodies from mouse A9 recipient cells and mouse A9 hybrids with paternal chromosome 15 *A9(15P)* clones and with maternal chromosome 15 *A9(15M)* clones. DNA from antibody-bound chromatin fractions was amplified by multiplex PCR using primer pairs for *G6pd* (*top band*) and *SNRPN* (*bottom band*)

monochromosomal hybrids as a resource for the efficient analysis of histone modifications. In contrast to our focus on the differentially methylated regions (DMR) of imprinted genes, previous reports were limited to regions that exhibit polymorphisms. It is well known that DMR play an essential role in the regulation of various imprinted genes and contain important *cis*-regulatory elements. We examined the CpG islands of *LIT1*, *H19*, and *SNRPN*, which have been demonstrated to be indispensable for imprinted expression of each gene (Huq et al. 1997; Dao et al. 1999; Horike et al. 2000), and found that histone modification of these regions was involved in allelic expression of imprinted genes. Thus, we can directly study the role of chromatin modifications around essential *cis*-regulatory elements responsible for the regulation of imprinting using our system.

We also examined the effect of TSA on the expression profiles of imprinted genes. In the A9 hybrids, TSA treatment reactivated the silent allele of the *LIT1* and *SNRPN* genes. Therefore, histone acetylation modulates not only the allelic chromatin modification of differentially methylated CpG islands but also the allelic expression of *LIT1* and *SNRPN*. Since methylated CpG islands recruit the mSin3A-histone deacetylase complex (Jones et al. 1998; Nan et al. 1998), we hypothesize that recruitment of this complex to the methylated CpG island on the maternal allele of *LIT1* and *SNRPN* causes repression through histone deacetylation accompanied by chromatin modification. On the other hand, we found that TSA treatment had no effect on the imprinted expression of the *H19* gene in our A9 hybrids. This finding is compatible with previous work by Pedone et al. (1999), who showed that a combination of reagents that inhibit both DNA methylation and histone deacetylation was required to activate the imprinted *H19* allele. A similar observation was made in the analysis of the corresponding region of the distal portion of chromosome 7 using methyltransferase-hypomorphic mice. This study showed that the *H19* imprint was erased fully, but the allelic bias at *Ipl*, *Impt1*, $p57^{kip2}$, and, to a lesser extent, *Kvlqt1* persisted in the region surrounding *Lit1* (Dao et al. 1999). The imprinted subdomain containing *H19* is subjected to particular methylation-hyperdependent mechanisms, which is in contrast to the other imprinted subdomain of 11p15.5 including *LIT1*. Actually, the imprinted expression of *H19* is regulated by the methylation-dependent binding of zing finger protein CTCF to an insulator region (Bell and Felsenfeld 2000; Hark et al. 2000).

The organization of chromatin structures influences chromosome function and epigenetic gene regulation. The growing appreciation of chromatin has led to the "histone code" hypothesis, in which modifications of histones constitute a boundary element for interaction with critical proteins to elicit the appropriate chromatin-based processes (Strahl and Allis 2000). Given that genomic imprinting is parent-of-origin-specific epigenetic gene regulation, the analysis of histone modifications that can distinguish the two parental alleles is essential. Although we applied a chromatin immunoprecipitation assay to analyze histone modifications, this technique also is useful to address questions concerning the in vivo regulation of the interactions between *cis*-regulatory elements and *trans*-acting factors in the context of chromosome structure and nuclear organization (Orlando 2000). Ren et al. (2000) showed that DNA

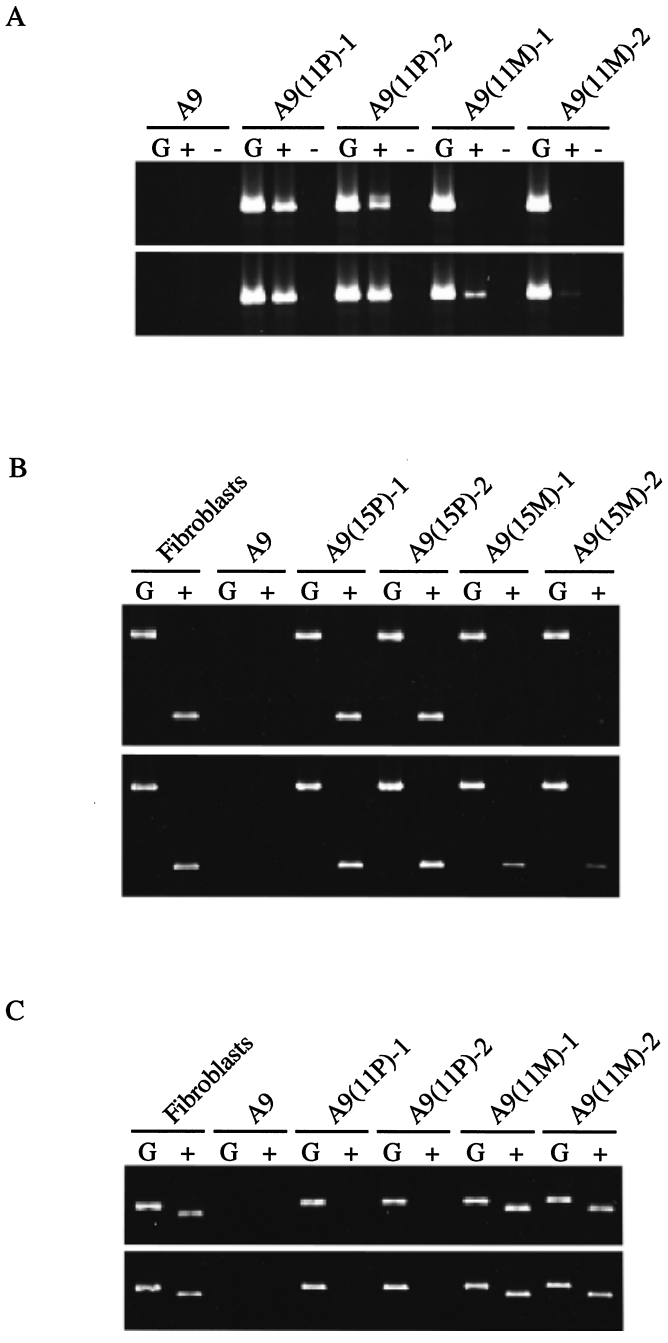

Fig. 3. A–C. Effect of histone deacetylase inhibitor. Expression profiles of imprinted genes, *LIT1* (**A**), *SNRPN* (**B**) and *H19* (**C**) were assessed by reverse transcriptase (RT)-PCR (+) in trichostatin A-untreated (*upper panels*) and -treated (*lower panels*) human fibroblasts, mouse A9 recipient cells, and A9 hybrid clones, each containing a paternal allele, *A9(11P)* or *A9(15P)*, or maternal allele, *A9(11M)* or *A9(15M)*. Genomic PCR was performed to verify the existence of the human chromosome in mouse A9 hybrids (G). RT-PCR products were not detected in reverse transcriptase-negative lanes (*minus sign*). Synthesis of first-strand cDNA was verified by PCR of *Gapdh* (data not shown)

fragments cross-linked to Gal4 or Ste12 were immunoprecipitated by specific antibodies, and the location of these DNA fragments were analyzed by DNA microarray. These genome-wide location analyses with expression profiles revealed how multiple functional pathways are coordinately controlled in vivo. In future applications, a differential display of the genome-wide location of DNA binding proteins between paternal and maternal A9 hybrids will facilitate investigation of gene regulatory networks and gene function in genomic imprinting. Thus, using these systems, we plan to clarify the regulation of imprinted genes.

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