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Report

Parent-of-Origin Specific Histone Acetylation and Reactivation of a Key Imprinted Gene Locus in Prader-Willi Syndrome

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To examine the chromatin basis of imprinting in chromosome 15q11-q13, we have investigated the status of histone acetylation of the *SNURF-SNRPN* locus, which is a key imprinted gene locus in Prader-Willi syndrome (PWS). Chromatin immunoprecipitation (ChIP) studies revealed that the unmethylated CpG island of the active, paternally derived allele of *SNURF-SNRPN* was associated with acetylated histones, whereas the methylated maternally derived, inactive allele was specifically hypoacetylated. The body of the *SNURF-SNRPN* gene was associated with acetylated histones on both alleles. Furthermore, treatment of PWS cells with the DNA methyltransferase inhibitor 5-azadeoxycytidine (5-aza-dC) induced demethylation of the *SNURF-SNRPN* CpG island and restoration of gene expression on the maternal allele. The reactivation was associated with increased H4 acetylation but not with H3 acetylation at the *SNURF-SNRPN* CpG island. These findings indicate that (1) a significant role for histone deacetylation in gene silencing is associated with imprinting in 15q11-q13 and (2) silenced genes in PWS can be reactivated by drug treatment.

Genomic imprinting refers to a parent-of-origin specific gene modification resulting in monoallelic expression of a gene dependent on its parental origin (Brannan and Bartolomei 1999). The molecular mechanism of genomic imprinting appears to share aspects of epigenetic regulation with X inactivation, since both phenomena involve DNA methylation and asynchronous replication of DNA (Heard et al. 1997). It is also well known that the active X chromosome in female mammals is associated with acetylated histones, whereas the inactive X is not (Jeppesen and Turner 1993). DNA methylation is involved in epigenetic marking of imprinted genes, and the methylated allele is usually transcriptionally silenced (Brannan and Bartolomei 1999). Recent studies have revealed a close association of DNA methylation and histone deacetylation in transcriptional silencing (Jones et al. 1998; Nan et al. 1998; Ng et al. 1999; Wade et al. 1999). Although a role for histone acetylation in the allelic expression of imprinted genes has been expected, specific evidence has only recently been demonstrated for the *H19/Igf2* imprinted genes (Hu et al. 1998; Pedone et al. 1999).

Human chromosome 15q11-q13 harbors multiple imprinted genes that are involved in Prader-Willi syndrome (PWS) (MIM 176270) and Angelman syndrome (AS) (MIM 105830), two distinct developmental and neurobehavioral disorders that provide an excellent model to study genomic imprinting (Nicholls et al. 1998). Of the candidate genes for PWS, the bicistronic *SNURF-SNRPN* locus appears to have a central role (Gray et al. 1999), since it is colocalized with an imprinting regulatory region in 15q11-q13 (Nicholls et al. 1998; Gray et al. 1999). The CpG island corresponding to the promoter region of *SNURF-SNRPN* is totally unmethylated on the paternally derived active allele, whereas it is completely methylated on the maternally derived inactive allele, and this imprint is very stable in normal cells (Nicholls et al. 1998). This study was designed to examine the chromatin basis for stable imprinting in 15q11-q13 and whether this state could be overcome.

To investigate a role of histone acetylation in imprinted gene regulation of *SNURF-SNRPN*, we used a

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chromatin immunoprecipitation (ChIP) assay (Braunstein et al. 1993; Coffee et al. 1999). ChIP was performed on the basis of a recommended protocol by the manufacturer of the anti-acetylated histone H3 and anti-acetylated histone H4 antibodies (Upstate Biotechnology). In brief, ~1 × 1^6 cells from lymphoblastoid lines from PWS patients with either a deletion of 15q11-q13 of paternal origin or maternal uniparental disomy (UPD), from AS patients with a deletion of 15q11-q13 of maternal origin, and from normal controls were treated with formaldehyde to cross-link DNA to protein in situ, sonicated to reduce the length of DNA, and immunoprecipitated with anti-acetylated histone H3 or anti-acetylated histone H4 antibodies. Immunoprecipitated DNA was dissolved in 100 μl TE, and 1 μl was used for the subsequent PCR reaction. Immunoprecipitated DNA thus represents DNA associated with acetylated histones and was detected by PCR with the use of a series of primer pairs specific for the *SNURF-SNRPN* locus (fig. 1*a*) as well as for the CpG island of the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) as a control. PCR was performed with the use of Amp Taq Gold (PE Biosystems), under standard conditions, with the following primer pairs for *SNURF-SNRPN*. A: 5′-GATGCTCAGGCGGGGATGTGTGCG-3′ (forward), 5′-GCTCCCCAGGCTGTCTCTTGAGAG-3′ (reverse); B: 5′-ACCTGAGACGAACTACAG-3′ (forward), 5′-TATTAGATGTGTCTGCTTCTTC-3′ (reverse); C: 5′-TAGGTGTCAGTTGTACCCGAGG-3′ (forward), 5′-GAATCAGATTCCTCGCTACTCC-3′ (reverse); D: 5′-TACTCTTTGAAGCTTCTGC-3′ (forward), 5′-CCTCAGCCTTATCATACAG-3′ (reverse); E: 5′-GTACCAGCTGGTGTGCCAA-3′ (forward), 5′-TCTAATGCCTGGTGGAGGGGG-3′ (reverse); and 5′-GCATCACCCGGAGGAGAAATCGG-3′ (forward), 5′-GTCACGTGTCGCAGAGGAGC-3′ (reverse) for *GAPDH*. *SNURF-SNRPN* primers A and *GAPDH* were amplified in the same reaction, whereas primers B–E were amplified independently (fig. 1*b*). PCR conditions for A and *GAPDH* were 94°C for 10 min, followed by 33 cycles of 94°C, 65°C, and 72°C each for 30 s. An annealing temperature of 55°C was used for B, C, and D, and of 60°C for E. PCR products were run on a 3% agarose gel and were visualized by staining with ethidium bromide.

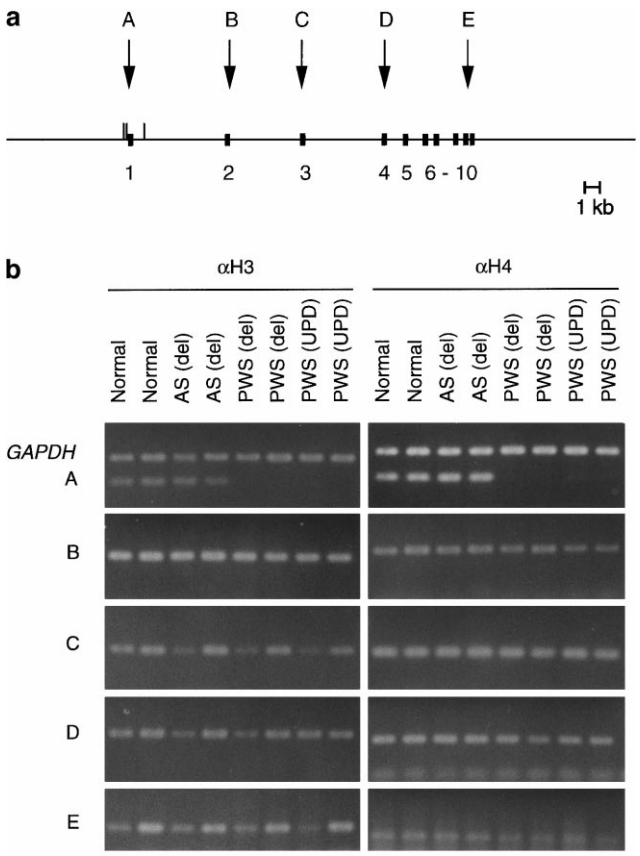

Figure 1 ChIP identifies parent-of-origin specific histone acetylation of the 5′ end of the *SNURF-SNRPN* gene locus. *A*, Structure of the *SNURF-SNRPN* gene and primer positions. A–E represent the positions of primer pairs. Exons are shown as numbered blackened boxes. Vertical bars around exon 1 represent *Not*I sites, indicating the CpG island. *B*, ChIP with the use of anti-acetylated H3 (αH3) and anti-acetylated H4 (αH4) antibodies detects parent-of-origin specific histone acetylation at the CpG island of *SNURF-SNRPN*. Primer positions are described in figure 1*a* and in the text. No amplification was detected for *SNURF-SNRPN* position A with either αH3 or αH4 immunoprecipitated DNA from PWS patients with a deletion of 15q11-q13 of paternal origin (PWS del) or with maternal UPD (PWS UPD), whereas clear amplification was detected for AS patients with a deletion of 15q11-q13 of maternal origin (AS del) and for normal controls. No such difference was demonstrated for *SNURF-SNRPN* position B–E.

Immunoprecipitated DNA with either anti-acetylated H3 or anti-acetylated H4 from all cell lines was readily amplified for *GAPDH* (fig. 1*b*). For the *SNURF-SNRPN* locus, however, no amplification was detected with primers A located in the CpG island for either anti-acetylated H3 or anti-acetylated H4 immunoprecipitated DNA from PWS patients with a deletion or with UPD, whereas clear amplification was detected for patients with AS and for normal controls (fig. 1*b*). This striking difference of amplification was only detected with primers A located in the CpG island of the *SNURF-SNRPN* locus, whereas primers B–E downstream of the CpG island did not demonstrate the difference for anti-acetylated H3 and for anti-acetylated H4 (fig. 1*b*). PWS patients with either a deletion or UPD have only the maternally derived *SNURF-SNRPN*, whereas AS patients with a deletion have only the paternally derived *SNURF-SNRPN;* normal controls have both. Therefore, these results indicate that the CpG island of the maternally derived inactive allele of *SNURF-SNRPN* is specifically associated with

hypoacetylation, whereas other regions of the maternally derived allele and the entire paternally derived active allele are associated with acetylated histones. These findings clearly indicate that *SNURF-SNRPN* is associated with parent-of-origin specific histone acetylation, which is confined to the CpG island that demonstrates parent-of-origin specific DNA methylation. The finding that hypoacetylation is confined to the CpG island of *SNURF-SNRPN* is consistent with recent findings that histone hypoacetylation is confined to the promoter regions of X-inactivated genes (Gilbert and Sharp 1999). The similar distribution of acetylated histones between the imprinted *SNURF-SNRPN* and X-inactivated genes further indicates that genomic imprinting and X inactivation may share similar silencing mechanisms.

Next, to further investigate the relationship between DNA methylation and histone acetylation, we treated the cell lines with the DNA methyltransferase inhibitor 5-azadeoxycytidine (5-aza-dC) (Chiurazzi et al. 1998). Approximately 3×10^5 cells were synchronized with 1 mM thymidine for two 8-h blocks and were subsequently incubated at 1 μM 5-aza-dC (Sigma) in RPMI1640 with 10% fetal calf serum for 7 d (Chiurazzi et al. 1998), then subjected to ChIP as well as to extraction of genomic DNA and total RNA. DNA methylation status at the CpG islands of *SNURF-SNRPN* and *GAPDH* was evaluated by methylation-PCR (M-PCR) with genomic DNA. M-PCR involves digestion of DNA (100 ng) with the methylation-sensitive restriction enzyme *Not*I (and with *Xba*I), ligation of a "bubble-anchor" oligonucleotide (Munroe et al. 1994) to *Not*I digested DNA, and PCR amplification with the use of an anchor-specific primer and a locus-specific primer flanking the *Not*I sites. With this method, only unmethylated DNA is amplified by PCR, whereas methylated DNA is not amplified. The double-stranded *Not*I bubble-anchor oligonucleotide was made by annealing with anchor top (5′-GGCCGAAGGAGAGGACGCTGTCTGTCGAAGGTAAACGGACGAGAGAAGGGAGAG-3′) and anchor bottom (5′-CTCTCCCTTCTACCCGGGAAGTTCGTCAACATAGCATTTCTGTCCTCTCCTTC-3′). One-twentieth of the ligation mixture was used as the PCR template. PCR primers used for M-PCR were *SNURF-SNRPN* A (reverse), *GAPDH* (reverse), and anchor-specific primer 5′-ACCCGGGAAGTTCGTCAACATAGCATTTCT-3′; and conditions were 94°C for 10 min, followed by 35 cycles of 94°C, 65°C, and 72°C for 30 s each. We have compared M-PCR on >100 individuals, including 60 patients with PWS, to conventional Southern blot analysis and the bisulfite-based methylation PCR (Kubota et al. 1997), with 100% concordance (unpublished data). Expression of *SNURF-SNRPN* or *GAPDH* was detected by RT-PCR. For RT-PCR, 1 μg of total RNA was reverse transcribed with Superscript II (Gibco BRL), by means of oligo dT, and one-tenth of the RT product was PCR amplified with primers for *SNURF-SNRPN* and *GAPDH,* as described (Nakao et al. 1994). Histone acetylation level at the CpG island of *SNURF-SNRPN* was evaluated by ChIP, as described above, with the use of primers for *GAPDH* and *SNURF-SNRPN* A. To quantitate levels of acetylation, ethidium bromide stained gel signals were captured by Bio Imaging System, BIS-202D (FUJI PHOTO FILM), and quantitative analyses were performed with National Institutes of Health Image software, version 1.61. The relative acetylation level of *SNURF-SNRPN* A was calculated by dividing signal intensity of *SNURF-SNRPN* A by that of *GAPDH*.

Treatment of the cell lines with 5-aza-dC induced a demethylation of the CpG island of *SNURF-SNRPN* in PWS patients with a deletion or with UPD as unmethylated allele-specific signals were clearly detected (fig. 2). RT-PCR demonstrated restored expression of *SNURF-SNRPN* in PWS patients with a deletion or with UPD after 5-aza-dC treatment, indicating reactivation of

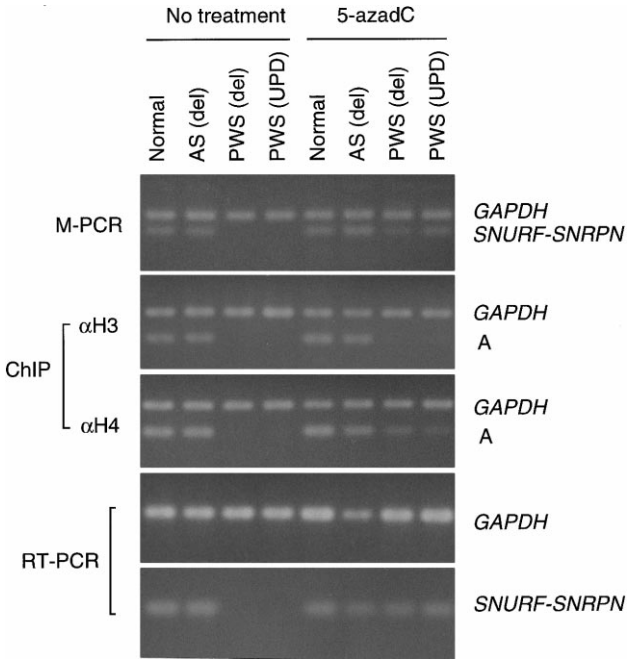

Figure 2 5-aza-dC treatment abolishes imprinting. Appearance of *SNURF-SNRPN* specific signals with M-PCR after 5-aza-dC treatment in PWS (del) and PWS (UPD) demonstrates demethylation of the CpG island of *SNURF-SNRPN*. ChIP signals for *SNURF-SNRPN* CpG island (position A) in both patients with PWS are significantly increased after 5-aza-dC treatment for αH4, but not significantly increased for αH3 (see text for quantitation). RT-PCR clearly demonstrates reactivation of *SNURF-SNRPN* in both patients with PWS after 5-aza-dC treatment.

SNURF-SNRPN (fig. 2). The histone acetylation level detected by ChIP in the CpG island of *SNURF-SNRPN* compared to *GAPDH* significantly increased from 0.02 ± 0.02 to 0.25 ± 0.13 ($n = 5$, $P = .023$) for acetylated H4 in the patients with PWS, whereas that for acetylated H3 remained unchanged (from 0.05 ± 0.03 to 0.05 ± 0.05 ($n = 5$, $P = .85$) (fig. 2). We also treated the cell lines with the histone deacetylase inhibitor, trichostatin A; however, it had no effect on histone acetylation, DNA demethylation, or expression of *SNURF-SNRPN* in PWS cell lines (data not shown). Promoter-specific hypoacetylation of *SNURF-SNRPN* may be due to recruitment of a histone deacetylase to the methylated DNA by methyl-cytosine binding proteins (Jones et al. 1998; Nan et al. 1998; Ng et al. 1999; Wade et al. 1999), or, alternatively, to specific factors recognizing the parent-of-origin specific imprint. Our finding of increased H4 acetylation after 5-aza-dC treatment supports the methylation-dependent recruitment of a histone deacetylase in silencing of the imprinted gene, because drug-induced demethylation was associated with an increase in histone acetylation as well as restoration of expression. Nonetheless, recent studies suggest an alternative possibility in which deacetylation may be required to remodel the chromatin prior to DNA methylation (Fuks et al. 2000). The colocalization of parent-of-origin specific histone acetylation and DNA methylation in the CpG island of *SNURF-SNRPN* provides evidence that both epigenetic events are integrally involved in regulating genomic imprinting in 15q11-q13. However, the different response of H3 and H4 for 5-aza-dC may indicate a specific histone H4 deacetylase involved in silencing mechanisms for imprinted genes.

Interestingly, our data clearly indicate that *SNURF-SNRPN* can be reactivated with 5-aza-dC. Patients with PWS have only the inactive maternally derived imprinted genes in 15q11-q13, which are epigenetically silenced although the genes are present. Therefore, reactivation of the genes may improve some aspects of the phenotype. Indeed, treatment with 5-aza-dC to increase the expression of the γ-globin gene has been demonstrated in patients with β-thalassemia (Ley et al. 1982). Although a more specific and nontoxic method of demethylation needs to be established, our findings demonstrate the potential for pharmaceutical treatment of imprinting-related disorders.

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Electronic-Database Information

The URL for data in this article is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for PWS [MIM 176270] and for AS [MIM 105830])

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