Role of histone acetylation and DNA methylation in the maintenance of the imprinted expression of the *H19* and *Igf2* genes

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Abstract ***H19*** **and** ***Igf2*** **are linked and reciprocally imprinted genes. We demonstrate that the histones associated with the paternally inherited and unexpressed** ***H19*** **allele are less acetylated than those associated with the maternal expressed allele. Cell growth in the presence of inhibitors of either histone deacetylase or DNA methylation activated the silent** ***Igf2*** **allele, whereas derepression of the silent** ***H19*** **allele required combined inhibition of DNA methylation and histone deacetylation. Our results indicate that histone acetylation as well as DNA methylation contribute to the somatic maintenance of** ***H19*** **and** ***Igf2*** **imprinting and that silencing of the imprinted alleles of these two genes is maintained via distinct mechanisms.**

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Key words: Histone acetylation; DNA methylation; Genomic imprinting; Insulin-like growth factor-2; H19; Histone deacetylase inhibitors

1. Introduction

A subset of genes present in diploid organisms display differential expression of the two alleles. Those whose expression is dependent on the parental origin of the allele are known as imprinted genes. The imprint is an epigenetic modification established during gametogenesis in a sex-specific manner, transmitted into the zygote and maintained in the embryo through cell generations, then erased in the germ line in order to be replaced by a new one. The nature of the imprint and the mechanism by which it causes preferential expression of one allele are still undefined. DNA methylation is certainly involved in at least one of the steps of the imprinting process, since imprinted genes show CpG sites which are differentially methylated on paternal and maternal alleles and mouse embryos deficient in DNA methyltransferase activity have lost genomic imprinting [1,2]. However, a few imprinted genes lack differential methylation or are unaffected by disruption of the DNA methyltransferase gene and many allele-specific methylations are not conserved throughout development, suggesting that other kinds of epigenetic modifications may contribute to this phenomenon as well.

In addition to DNA methylation, another epigenetic modification that may modulate transcription in the chromatin context is histone acetylation. Lysines at the N-terminal tails of core histones may be acetylated, particularly within nucleosomes associated with transcribed genes [3]. Conversely, inactive genomic regions, such as the silent X chromosome and constitutive heterochromatin, are associated with hypoacetylated histones. It has been suggested that acetylation alters nucleosome conformation by decreasing the affinity of histones for DNA or causes a change in the higher-order chromatin structure by interfering with the internucleosomal contacts [4]. DNA hypermethylation can cause a decrease in the level of histone acetylation [5], consistent with recent demonstrations of an interaction between the methyl binding protein MeCP2 and histone deacetylase [6,7].

A cluster of imprinted genes is located on the distal portion of mouse chromosome 7 and the homologous human region on chromosome 11p15.5 [8]. The insulin-like growth factor-2 (*Igf2*) and *H19* genes are located in this region, separated by 75 kb and imprinted in a reciprocal manner. *Igf2* is expressed exclusively from the chromosome of paternal origin and *H19* is expressed only from the maternal allele. The *H19* gene is included in a 7–9 kb region, which is hypermethylated on the inactive allele, and its promoter displays an open chromatin conformation only on the expressed allele [9,10]. In contrast to the differential methylation of the *H19* gene, only limited differences in the extent of methylation at a few CpG sites distant from the promoters and similar nuclease sensitivity have been found between paternal and maternal alleles of the *Igf2* gene [11–13]. The expression of the *Igf2* and *H19* genes is linked and dependent on common regulatory elements [14,15].

In this paper, we show that differentially acetylated histones are associated with paternal and maternal alleles of the *H19* gene in cultured mouse fibroblasts, such that the silent allele is hypoacetylated. No differences in the relative histone acetylation levels of the two parental *Igf2* alleles were found in the 3′ untranslated region (UTR) and 700 bp 5′ of promoter P2. However, treatment of the cells with histone deacetylase inhibitors caused relaxation of *Igf2* imprinting, an effect that was additive to that obtained with the cytosine methylation inhibitor 5-aza-2′-deoxycytidine (5-azaC). Little or no effect of the histone deacetylase or DNA methylation inhibitors alone was observed on *H19* imprinting, but the combination of both did reactivate the silent *H19* allele. These results in-

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Abbreviations: Igf2, insulin-like growth factor-2; 5-azaC, 5-aza-2′-deoxycytidine; TSA, trichostatin; UTR, untranslated region; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; RFLP, restriction fragment length polymorphism

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dicate that both DNA methylation and histone acetylation contribute to the maintenance of the imprinting status of the *H19* and *Igf2* genes and that inhibition of both types of epigenetic modifications is required to activate the imprinted *H19* allele.

2. Materials and methods

2.1. Cell cultures

Mice of BALB/c and C57-Black6 strains were obtained from Charles River Italia. The cells were derived from a 3-day-old animal generated from a cross between a BALB/c father and a Black6 mother. Primary cultures of fibroblast cells were obtained as previously described [16]. The expression of *Igf2*/*H19* genes in these cells was estimated to be at least 50-fold higher than that of adult liver and about one tenth that of fetal (E20) liver (data not shown). *Igf2*/*H19* expression was maintained in these cultured cells at least until the tenth passage. For the analysis of histone deacetylase inhibitors, fourth- or fifth-passage cells were plated at a seeding density of 5×10^5 cells/dish. Two days after seeding, culture medium was replaced with medium containing the histone deacetylase inhibitors sodium butyrate or trichostatin A (TSA) at the indicated concentrations. Twenty-four hours later, RNA was harvested from the cells. In order to test the effect of 5-azaC and 5-azaC plus histone deacetylase inhibitors, fifth-passage cells were plated at a density of 4×10^5 cells/dish. Twenty-four hours later, 100 or 200 μM 5-azaC was added to the cells. Medium with freshly added 5-azaC was changed on the third and fifth day of culture. On the sixth day, where indicated, 100 ng/ml TSA or 50 mM sodium butyrate was added still in the presence of 5-azaC. Twenty-four hours later all the cells were harvested and analyzed.

2.2. Histone acetylation analysis

Nuclei for chromatin immunoprecipitation were prepared by lysing cells in 10 mM Tris, 10 mM NaCl, 3 mM $MgCl_2$, and 0.4% NP-40. Nuclei were washed and digested in the same buffer with micrococcal nuclease for 10 min at a concentration experimentally determined to give a mostly mononucleosomal chromatin preparation. 100 μg of chromatin prepared in this way was immunoprecipitated with 20 μg of antibody (Upstate Biotechnology, Lake Placid, NY, USA) against acetylated H3 or H4 tail peptides as described [17]. The antibody recognized di- and tetraacetylated lysine epitopes of histones H3 and H4, respectively. In a control experiment without primary antibody, only a small fraction of the DNA obtained with either anti-acetylated H3 or H4 antibody added was pulled down with the protein A agarose beads (data not shown). Differential allelic acetylation was detected by quantitative amplification of 5 ng of immunoprecipitated DNA using PCR primers which distinguish the BALB/c and Black6 haplotypes either directly as a length polymorphism or by restriction enzyme digestion following amplification. In all reactions, a trace amount of one ^{32}P-end labelled primer was included and visualization of amplified products took place on a phosphorimager, allowing relative allelic intensities to be measured.

Immunoprecipitated DNA at the 3′ position of the *Igf2* gene was detected by amplification with primer pairs Igf2-C (5′-GATTATACTCACACCACAGGC-3′) and Igf2-D (5′-CATCCAATTATGTGGGTGTGC-3′) followed by separation on an 8% denaturing polyacrylamide sequencing gel, allowing detection of paternal and maternal products which are approximately 115 bp in length but differ by 8–12 bp [18]. Analysis of the *H19* promoter region was performed by amplification with primers H19-C (5′-GGCAGGATAGTTAGCAAAGG-3′) and H19-B (5′-CCTCAGTCTTTACTGGCAAC-3′). Products of the *H19* amplification were incubated with the restriction endonuclease *Msp*I, which produces a radiolabelled 76 bp digested paternal fragment and leaves a 139 bp undigested maternal band following electrophoresis as above [19]. DNA from the two alleles at the *Igf2* promoter region was amplified with the primer pair Igf2-E (5′-GGTTTGAGCTACGTTTCCCG-3′) and Igf2-F (5′-ATCTCCTAGCAGCCTTTGGG-3′) and digestion of the products with *Ava*II restriction endonuclease. Electrophoresis of the *Ava*II digest separated a 74 bp digested maternal fragment and a 149 bp undigested paternal band. All PCRs were carried out in 5 μl reactions using an initial denaturation at 94 C for 1 min, then 35 cycles at 94°C, 30 s; 62°C, 30 s; and 72°C, 30 s. As controls for amplification, analyses were also performed on purified parental BALB/c and C57-Black6 DNA. Additionally, control reactions were performed on ranges of stoichiometric mixtures of parental DNAs to ensure that the ratios of final products detected accurately reflected the relative concentrations of the alleles in the input DNA.

2.3. Isolation of DNA and RNA

Genomic DNA was prepared from cells by proteinase K digestion and phenol-chloroform extraction methods [20]. Total RNA was isolated using the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method [21].

2.4. RT-PCR analysis

For detection of the expressed alleles, 0.5–1 μg total RNA was first DNase I treated, and then reverse transcribed using 200 units of Superscript RT (BRL) in the presence of 0.4 μg random hexamers (Boehringer), 10 mM DTT, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM dNTP-lithium salt (Boehringer) and 20 units of RNase inhibitor (Promega). Incubation was at 37°C for 10 min, followed by 1 h at 42°C. One fiftieth of first strand cDNA was used in the PCR reaction. To test for contamination by genomic DNA, all RNA samples were run in duplicate without addition of reverse transcriptase.

The $(CA)_n$ repeat polymorphism of the mouse *Igf2* gene [18] was analyzed using the primers Igf2-A (5′-GAACTTAATTGGCACAAGCCC-3′) and Igf2-B (5′-ACCATGCAAACTGCTCAGGA-3′) or Igf2-C and Igf2-D (see above). The PCR reaction was carried out in 25 μl with 1 unit Taq DNA polymerase (Boehringer) using an initial denaturation step of 2 min at 95°C followed by 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min for primers A and B and 94°C for 30 s, 63°C for 30 s and 72°C for 30 s for primers C and D. In both cases, a final elongation of 5 min at 72°C terminated the reactions. For allele visualization, PCR products were electrophoresed on denaturing 8% polyacrylamide-urea gel after including a ^{32}P-end labelled primer in the amplification.

The *Msp*I RFLP of the *H19* gene [19] was analyzed by PCR amplification using primers H19-A (5′-CACTGACCTTCTAAACGAGG-3′) and H19-B (above) chosen from the nucleotide sequence of the mouse gene [22]. Conditions with primers A and B were an initial denaturation step of 2 min at 95°C followed by 36 cycles at 94°C for 30 s, 63°C for 30 s and 72°C for 30 s and a final extension at 72°C for 5 min; the PCR products were internally labelled by performing the PCR reactions in the presence of [α-^{32}P]dGTP (Amersham). For allele visualization, the PCR products were digested with *Msp*I and separated on non-denaturing 6% polyacrylamide gels.

All the numerical values were obtained by computer quantitation of the image using a Molecular Dynamics phosphorimager.

3. Results

3.1. Relative levels of histone acetylation on paternal and maternal alleles of the H19 and Igf2 genes

In order to study the role of histone acetylation in the expression of the *H19* and *Igf2* genes we cultured fibroblasts (C×B) from a 3-day-old C57-Black6 (maternal)×BALB/c (paternal) mouse and analyzed the relative levels of histone acetylation on the paternal and maternal alleles in immunoprecipitated chromatin. Chromatin was prepared from the C×B cells and immunoprecipitated with antibodies raised against acetylated amino-terminal tails of histone H3 or H4. Fragments ranging from 115 to 150 bp and containing sequences which were polymorphic between BALB/c and C57-Black6 strains were amplified by PCR and used to determine the relative amounts of paternal and maternal alleles in the immunoprecipitated nucleosomes (Fig. 1A). Control reactions performed on ranges of stoichiometric mixtures of parental DNAs ensured that, under the PCR conditions used, the ratios of final products detected accurately reflected the relative concentrations of the alleles in the input DNA (Fig. 1B–D).

We first analyzed the chromatin acetylation of a region

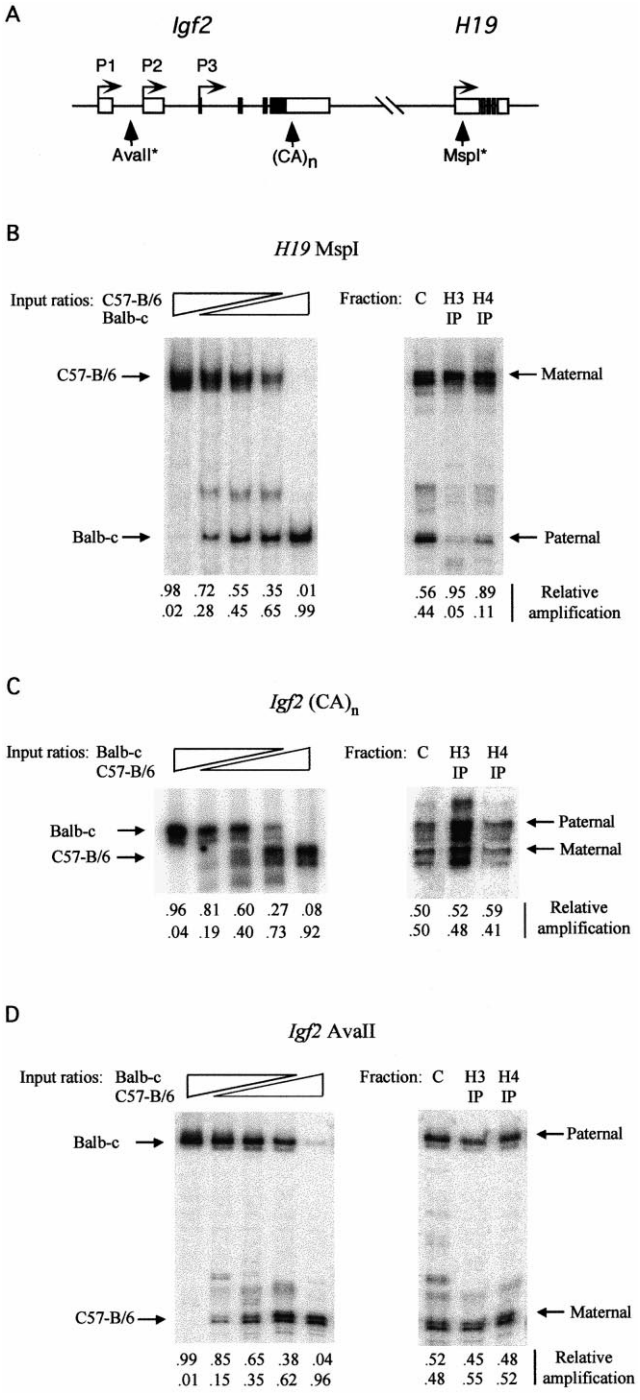

Fig. 1. Relative histone acetylation levels of the paternal and maternal alleles of the *H19* and *Igf2* genes. A: Structure of the mouse *Igf2/H19* region and location of polymorphisms. Exons are shown as filled boxes. Transcription start sites are indicated by bent arrows. The $(CA)_n$ polymorphism and the RFLPs utilized in this study are indicated by vertical arrows. B–D: Histone acetylation assay. Different input ratios of parental C57-Black6 and BALB/c DNAs or DNA of C×B cells prepared from non-immunoprecipitated, micrococcal nuclease-digested chromatin (C) or nucleosomes immunoprecipitated with monoclonal antibody directed against acetylated histone H3 (H3 IP) or H4 tails (H4 IP) were subjected to PCR with primers specific for the *H19 Msp*I RFLP (B), the *Igf2* $(CA)_n$ repeat (C) or the *Igf2 Ava*II RFLP (D). One radiolabelled primer of each pair was included in the PCR. The paternal and maternal alleles were separated on denaturing 8% polyacrylamide-urea gels and are indicated by arrows. Relative amplification of the parental alleles was calculated by computer quantitation of the gel following exposure to a phosphorimager.

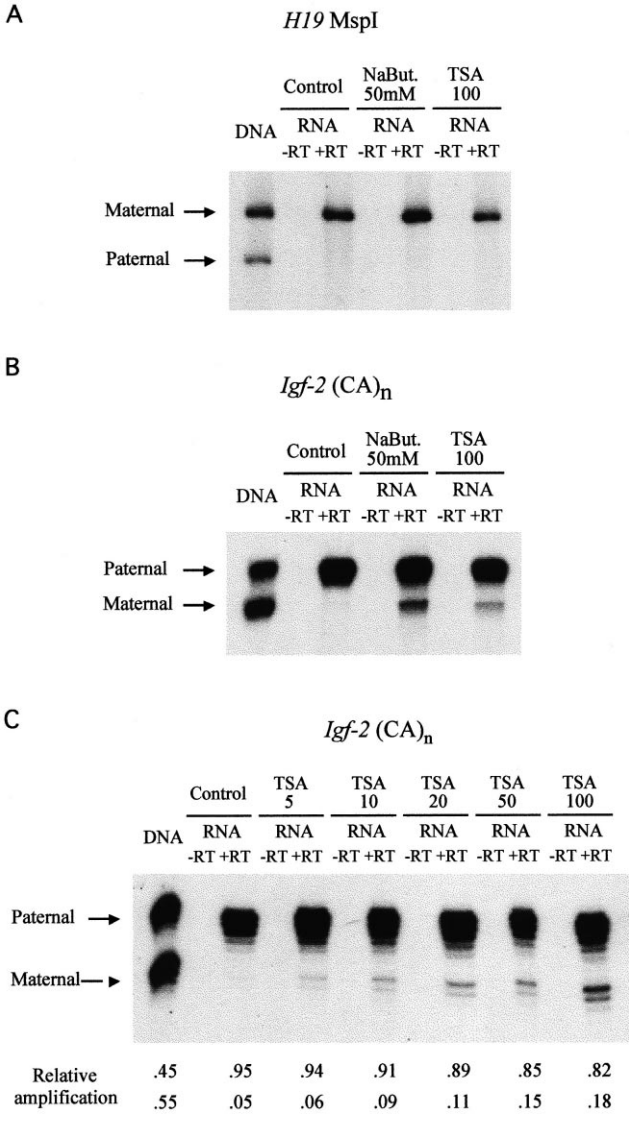

Fig. 2. Effect of inhibition of histone deacetylase activity on the imprinting of the *H19* and *Igf2* genes. A: Analysis of the allele-specific expression of the *H19* gene. RNA was purified from untreated C×B cells (control) and cells treated with the histone deacetylase inhibitors sodium butyrate (50 mM) or TSA (100 ng/ml), used for cDNA synthesis and subjected to PCR amplification. The PCR products obtained from genomic DNA and cDNAs with the primers H19-A and H19-B were digested with the polymorphic restriction enzyme *Msp*I and analyzed on 6% acrylamide gel. B: Analysis of the allele-specific expression of the *Igf2* gene. Genomic DNA and cDNAs prepared as in A were PCR amplified with the primers Igf2-A and Igf2-B and electrophoresed on denaturing 8% polyacrylamide-urea gel. C: Dose-dependent effect of TSA on *Igf2* allelic expression. DNA and cDNA prepared from cells treated with increasing doses of TSA (5, 10, 20, 50 and 100 ng/ml) were PCR amplified with the primers Igf2-C and Igf2-D. To test for contamination by genomic DNA, all RNA samples were run in duplicate with (+RT) or without (−RT) the addition of reverse transcriptase.

spanning a polymorphic *Msp*I site present in the first exon of the *H19* gene (Fig. 1A and [19]). Equivalent amounts of paternal and maternal *H19* alleles were amplified from the non-immunoprecipitated nucleosomes (Fig. 1B, lane Co). However, the maternal, transcriptionally active *H19* allele was preferentially amplified with respect to the paternal allele in the samples immunoprecipitated with both anti-acetylated H3

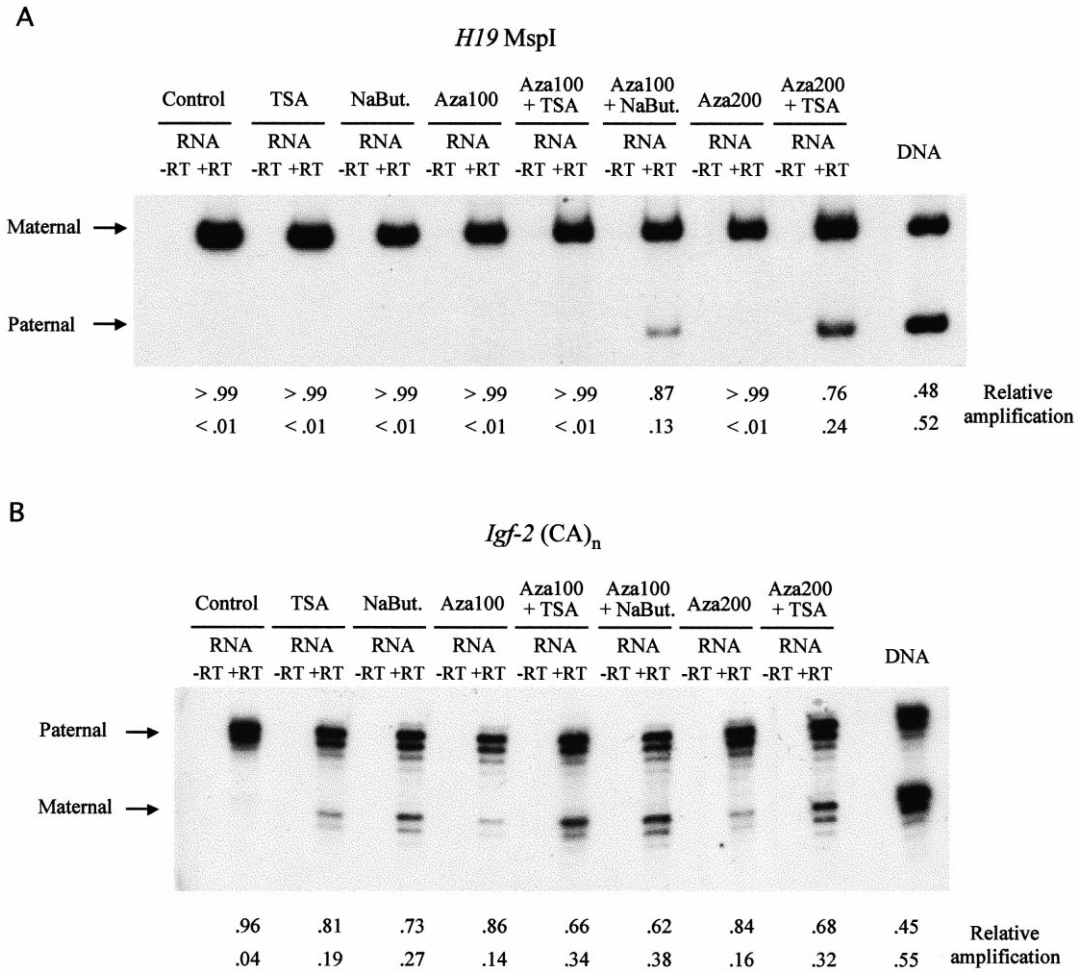

Fig. 3. Effect of inhibition of DNA methylation and histone deacetylase activity on the imprinting of the *H19* and *Igf2* genes. Analysis of the allele-specific expression of the *H19* (A) and *Igf2* (B) genes in untreated C×B cells (control), and cells treated with the histone deacetylase inhibitors sodium butyrate (50 mM) or TSA (100 ng/ml) alone, with the cytosine methylation inhibitor 5-azaC at a concentration of 100 μM (Aza 100) and 200 μM (Aza 200), and with the combination of 5-azaC and sodium butyrate or 5-azaC and TSA. The paternal and maternal alleles of the *H19* and *Igf2* genes were analyzed as in Fig. 2A,C.

and H4 antibodies (Fig. 1B, lanes H3 IP and H4 IP). Therefore, a strong difference exists in the relative acetylation level of the two parental *H19* alleles in the amplified region, with the silent paternal allele being hypoacetylated.

To investigate the chromatin acetylation state of the *Igf2* gene, we first analyzed the 3′ UTR, where a polymorphic $(CA)_n$ repeat has been described between the BALB/c and the C57-Black6 strains (Fig. 1A and [18]). As shown in Fig. 1C, the paternal and maternal *Igf2* alleles were amplified with similar efficiency from control and immunoprecipitated samples, indicating that, at least in the 3′ UTR, both the active and silent *Igf2* alleles are acetylated at comparable levels.

Because the 3′ UTR polymorphic $(CA)_n$ repeat is located at the 3′ terminus of the transcript, we looked for polymorphisms closer to the transcription start site. The murine *Igf2* gene is transcribed from two stronger (P2 and P3) and one weaker promoter (P1, see [23]). A polymorphic *Ava*II site was found 705 bp upstream of the start site of promoter P2 (Fig. 1A) which allowed analysis of histone acetylation in this region. As with the 3′ UTR, paternal and maternal alleles were similarly immunoprecipitated by anti-acetylated H3 and H4 antibodies (Fig. 1D), indicating that no major difference exists in the acetylation status between the active and silent *Igf2* alleles also in the region immediately upstream of promoter P2.

3.2. Effect of inhibition of histone deacetylases

The relevance of histone acetylation in determining the relative transcriptional activity of the paternal and maternal alleles of the *H19* and *Igf2* alleles was tested by analyzing the effects of the histone deacetylase inhibitors TSA and sodium butyrate on the imprinting status of these genes in cultured C×B cells.

RT-PCR amplification of the polymorphic sequences present in the *H19* and *Igf2* transcripts showed that untreated C×B cells maintained the imprinting of the *H19* and *Igf2* genes, as in the animal of origin (Fig. 2A,B, control lanes). Treatment of the cells with 50 mM sodium butyrate or 100 ng/ml TSA did not alter the imprinted expression of the *H19* gene (Fig. 2A). In contrast, the same treatments caused a significant relaxation of *Igf2* imprinting, since both histone deacetylase inhibitors reactivated the normally silent maternal *Igf2* allele (Fig. 2B). As shown in Fig. 2C, the effect of TSA on *Igf2* imprinting was dose-dependent.

3.3. Effect of inhibition of DNA methylation

The heavily methylated status of the maternal *H19* allele observed in tissues and cultured cells, including the C×B strain ([9,10,24] and data not shown), prompted us to investigate the effect of coupling the inhibition of histone deacetylase activity to inhibition of DNA methylation. For this purpose, C×B cells were incubated with increasing concentrations of the cytosine methylation inhibitor 5-azaC associated or not with the histone deacetylase inhibitors and the relative expression of paternal and maternal *H19* and *Igf2* alleles was determined by RT-PCR, as described above.

Little or no effect of 5-azaC treatment alone on the imprinting status of the *H19* gene was observed (Fig. 3A). However, combinations of 100 μM 5-azaC and 50 mM sodium butyrate or 200 μM 5-azaC and 100 ng/ml TSA caused a significant activation in the expression of the paternal relative to the maternal *H19* allele (Fig. 3A). Unlike the *H19* gene, a limited activation of the imprinted *Igf2* allele was observed in C×B cells also after treatment with 5-azaC alone, whereas a more dramatic relaxation of *Igf2* imprinting was achieved by 5-azaC and sodium butyrate or TSA together (Fig. 3B).

Overall, these data show that inhibition of both DNA methylation and histone deacetylation in C×B cells caused reactivation of the silent maternal allele of the *Igf2* gene and that the two effects were additive. Additionally, treatment with either DNA methylation or histone deacetylase inhibitors alone did not affect *H19* imprinting, but the two kinds of chemicals together had a synergistic effect on the activation of the imprinted allele of the *H19* gene.

4. Discussion

Heritable epigenetic modifications must control the unequal expression of paternal and maternal alleles of the imprinted genes in somatic tissues. Hypermethylation of the silent alleles occurring on most, although not all, imprinted genes is likely part of the mechanism involved in the maintenance of the imprinting status, consistent with the suppressing activity of this DNA modification on gene expression. Here we show that the two alleles of the imprinted *H19* gene display different levels of histone acetylation in the region of the first exon. The unexpressed and hypermethylated allele is associated with histones with low levels of lysine acetylation. Conversely, chromatin at the expressed and undermethylated *H19* allele is hyperacetylated. Therefore, like heterochromatin and the inactive X chromosome, the imprinted *H19* allele has the typical features of transcriptionally inactive DNA, i.e. CpG hypermethylation, closed chromatin conformation, and histone hypoacetylation. Svensson et al. [25] showed, by the allele-specific in situ hybridization technique, that treatment of embryos with the histone deacetylase inhibitor TSA reactivated in a variegated manner the imprinted *H19* allele in the extraembryonic but not embryonic cells. We found that growth in the presence of TSA alone did not exert a significant effect on *H19* imprinting in a population of cultured somatic cells. Rather, a combination of reagents that counteracts both cytosine methylation and histone deacetylation is required to activate the imprinted *H19* allele. These results indicate that both epigenetic modifications participate in maintenance of the imprinting status of this gene and that alleviation of either modification individually is insufficient to allow reactivation of the imprinted *H19* allele in somatic cells.

The *Igf2* gene does not display major allele-specific differences in the level of histone acetylation, at least in the two 5′ flanking and 3′ untranslated regions analyzed, but inhibition of histone deacetylase activity causes relaxation of its imprinting status. Recently, Hu et al. [26] have reported a similar effect of TSA and sodium butyrate on *Igf2* imprinting in human cells. Although these results clearly indicate that histone acetylation is involved also in the control of *Igf2* imprinting, they do not give any clue on how this is achieved. The complex mechanism of regulation of this gene may possibly explain these findings. *Igf2* shows only limited allele-specific differences in CpG methylation; unlike *H19*, methylation at these sites is associated with the expressed rather than the silent allele and it appears to be dispensable for gene expression [11,12,27]. Two *cis*-acting regulatory elements located near *H19* have been demonstrated so far to control the expression of the *Igf2* gene. The more 3′ one is an endoderm-specific enhancer activating *Igf2* on the paternal chromosome and *H19* on the maternal homolog [14]. The second element (differentially methylated domain, DMD) is a region located 5′ of the *H19* gene, whose methylation status is preserved throughout development on the maternal chromosome and whose presence is required for both *Igf2* and *H19* imprinting [15]. Two models have been proposed for how these two elements operate [2]. According to the first, the promoters of the two genes compete for the same enhancer element(s) and the methylation status of the *H19* promoter determines which gene is activated. The second model suggests that the DMD is a chromatin insulator, which, if not methylated, interferes with the activation of the *Igf2* gene by the enhancer. In both models, the inherited methylation status of the DMD could be reflected in the methylation state of the *H19* promoter as well, causing suppression of the maternal *H19* allele. The absence of major allele-specific differences in the histone acetylation level of the nucleosomes associated with the *Igf2* gene is consistent with the finding of open chromatin conformations on both parental alleles [11] and is one additional line of evidence indicating that the expression of the *Igf2* alleles is dependent on regulatory elements located far from its transcribed sequence. The histone deacetylase inhibitors could interfere with the acetylation status of the nucleosomes associated with distal elements rather than that of nucleosomes over the *Igf2* promoters or coding region itself. Alternatively, differential acetylation of paternal and maternal alleles could be limited to very few nucleosomes proximal to the transcription start site, to only some of the lysine residues recognized by our antibodies, could target proteins other than histones or affect other gene functions which indirectly control the imprinting status [28–31]. Regulatory sequences located distal to the *Igf2* gene may also be involved in mediating the effect of 5-azaC on *Igf2* imprinting, since the maternal *Igf2* allele appears demethylated even in its naturally silent state [11]. Consistent effects of DNA methylation inhibitors on *Igf2* expression have been reported by Eversole-Cire et al. [32] and Hu et al. [33].

Recent evidence indicates that deregulation of histone acetylase and deacetylase activity in human cancer is a frequent event [34]. Relaxation of the imprinting status, resulting in the doubling of the active gene dosage of the *Igf2* gene, has also been observed in a high number of human neoplasms [35–38].

Our results suggest that the inhibition of the histone deacetylases or the inappropriate activation of the histone acetyltransferases could be the mechanism causing the loss of *Igf2* imprinting in human cancer. Also, the observed irreversibility of the *H19* imprinting in the presence of histone deacetylase inhibitors alone in cultured mouse cells is consistent with the infrequent occurrence of loss of *H19* imprinting in human cancer [36,39]. IGF-II, the protein product of the *Igf2* gene, is an important mitogen for tumor cells [40] and elucidation of the mechanisms contributing to its activation may help find new strategies for cancer therapy.

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