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Inhibition of Histone Deacetylases Alters Allelic Chromatin Conformation at the Imprinted *U2af1-rs1* Locus in Mouse Embryonic Stem Cells*

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Most loci that are regulated by genomic imprinting have differentially methylated regions (DMRs). Previously, we showed that the DMRs of the mouse *Snrpn* and *U2af1-rs1* genes have paternal allele-specific patterns of acetylation on histones H3 and H4. To investigate the maintenance of acetylation at these DMRs, we performed chromatin immunoprecipitation on trichostatin-A (TSA)-treated and control cells. In embryonic stem (ES) cells and fibroblasts, brief (6-h) TSA treatment induces global hyperacetylation of H3 and H4. In ES cells only, TSA led to a selective increase in maternal acetylation at *U2af1-rs1*, at lysine 5 of H4 and at lysine 14 of H3. TSA treatment of ES cells did not affect DNA methylation or expression of *U2af1-rs1*, but was sufficient to increase DNase I sensitivity along the maternal allele to a level comparable with that of the paternal allele. In fibroblasts, TSA did not alter *U2af1-rs1* acetylation, and the parental alleles retained their differential DNase I sensitivity. At *Snrpn*, no changes in acetylation were observed in the TSA-treated cells. Our data suggest that the mechanisms regulating histone acetylation at DMRs are locus and developmental stage-specific and are distinct from those effecting global levels of acetylation. Furthermore, it seems that the allelic *U2af1-rs1* acetylation determines DNase I sensitivity/chromatin conformation.

The allelic expression of imprinted genes in mammals depends on whether the allele is inherited from the mother or the father (1). Genetic experiments have established that allelic differences in DNA methylation at CpG dinucleotides are essential for the correct expression of most imprinted genes (2). The great majority of imprinted loci have defined regulatory sequences that are methylated predominantly on one or other of the two parental alleles. At several of these differentially methylated regions (DMRs),[1] the allelic methylation is established in the germ line and is maintained during embryonic and postnatal development (reviewed in Ref. 3). However, CpG methylation cannot be the sole determinant in the somatic maintenance of imprints. At constitutive DMRs, allelic methylation patterns must somehow be protected from the genome-wide demethylation that occurs following fertilization and during early stages of development (reviewed in Ref. 4). Although some mouse DMRs lose methylation during preimplantation development, they can regain allelic methylation patterns at later stages (3). To account for such observations, it has been argued that the somatic maintenance of epigenetic marks at DMRs may involve multiple, interdependent, modifications including DNA methylation, nonhistone protein binding, and alterations to nucleosomes and chromatin (5–7).

Chromatin appears to be organized differently at the maternal and paternal alleles of DMRs. Several DMRs, for instance, display differential chromatin compaction when assayed by enzymatic digestion in nuclei (5). Along the splice factor-encoding, imprinted *U2af1-rs1* gene on mouse chromosome 11 (8, 9), the methylated and repressed maternal allele is severalfold more resistant to DNase I than the unmethylated, active, paternal allele (10). It has been suggested that the DMR comprising exon 1 of the human *SNRPN* gene on chromosome 15q11-q13 (11) also has differential chromatin compaction, based on assays that map matrix attachment regions (12). This DMR corresponds to the imprinting control center involved in the neuro-developmental Prader-Willi Syndrome (13, 14); and both in humans and mice, it shows increased histone H3 and H4 acetylation on the unmethylated paternal allele (15, 16). Histone H4 associated with the differentially methylated region-2 of the IGF2 receptor gene (*Igf2r*) on mouse chromosome 17 is heavily acetylated on the unmethylated paternal allele and underacetylated on the maternal allele (17). The DMR encompassing the imprinted *U2af1-rs1* gene also shows pronounced acetylation differences between the methylated maternal and the unmethylated paternal allele. By using antisera specific for particular acetylated lysines on histones H3 and H4, we previously established that the underacetylation of H4 at the methylated *U2af1-rs1* allele is confined to lysine 5, whereas for H3, at least three of the four acetylatable lysine residues were

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF309654.

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[1] The abbreviations used are: DMR, differentially methylated region; TSA, trichostatin-A; HDAC, histone deacetylase; ES, embryonic stem; ChIP, chromatin immunoprecipitation; SSCP, single strand conformational polymorphism; UTR, untranslated region; MBD, methyl-CpG-binding domain.

underacetylated. Similar results were obtained for the constitutive DMR comprising exon 1 of the mouse *Snrpn* gene (16). Furthermore, we found that by inducing high levels of CpG methylation on the paternal *U2af1-rs1* allele, we could bring about underacetylation of H3, but not of H4, lysine 5 (16). Thus, allelic differences in histone acetylation can be both histone-specific and lysine residue-specific and can be linked differently to CpG methylation.

In the present study we explore how allelic patterns of histone acetylation are maintained at the DMRs of the imprinted mouse genes *U2af1-rs1* and *Snrpn* by examining the *in vivo* effects of trichostatin-A (TSA), a highly specific inhibitor of histone deacetylases (18). We used ES cells and differentiated cells derived from interspecific hybrid embryos to compare directly the maternal and paternal alleles of the *U2af1-rs1* and *Snrpn* genes in chromatin and expression assays. This analysis demonstrates that the DMRs at the imprinted *Snrpn* and the *U2af1-rs1* genes are highly resistant to TSA treatments that cause global hyperacetylation of histones H3 and H4. In undifferentiated ES cells, but not in embryonic fibroblasts, and at the *U2af1-rs1* DMR only, TSA treatment induces selective, lysine residue-specific changes in acetylation. These changes are associated with altered chromatin conformation along this imprinted locus.

EXPERIMENTAL PROCEDURES

Mice, Cells, and in Vitro Culture—Mice that were maternally (Matdi11) or paternally (Patdi11) disomic for chromosome 11 were produced by intercrossing animals heterozygous for the Robertsonian translocation Rb(11.13)4Bnr (19). Primary embryonic fibroblasts were derived from day 14 fetuses (line EF1; Ref. 16) and were cultured in DMEM medium containing 20% fetal calf serum. For chromatin assays, early passage (<passage 5) EF1 fibroblasts were used. ES line SF1-1 was cultured in ES medium with 10^3 units/ml of leukemia inhibitory factor (LIF) (20). For chromatin studies, semiconfluent early passage (<passage 15) SF1-1 cells were used that were morphologically undifferentiated. For TSA treatment, exponentially growing cells were cultured for 6 h in medium supplemented with TSA (at 300 nM).

Nuclease Sensitivity Assays, Southern and Northern Hybridization, and Reverse Transcriptase PCR—Nuclei were isolated from tissue or cultured cells and were resuspended in DNase I, MNase, or *Msp*I digestion buffer at ~10^7 nuclei/ml, as described previously (10). For the DNase I assay, 200-μl aliquots of nuclei suspension were incubated for 10 min at 25 °C at increasing concentrations of enzyme (Roche Molecular Biochemicals). DNA extraction and Southern hybridization were performed as described previously (10). Hybridized filters were analyzed by phosphorimager (FLA3000, Fuji) and intensities were determined using the Quantity-One imaging software (Bio-Rad). Probe 7 is a 397-bp fragment (nucleotides 3556–3953 of the sequence for GenBank™ accession number AF309654) and probe 8 is 389-bp (nucleotides 7336–7725 of the sequence for GenBank™ accession number AF309654). For reverse transcriptase PCR analysis, poly(A)$^+$ RNA was extracted from cells using a Qiagen "Oligotex direct mRNA kit." First strand DNA synthesis was from 100–200 ng of mRNA using random primers and Superscript II reverse transcriptase (Invitrogen). cDNA was used as template for *U2af1-rs1* amplification (forward, cgcagatcagacatactgcgg; reverse, tgtggtacggccagcctatg) and *Snrpn* amplification (forward, gagagggagccggagatg; reverse, ttgctgttgctgagaacgtc) in a mixture containing [α-^{32}P]dCTP, and the resulting products were migrated through an SSCP gel. For total RNA extraction we used a Qiagen "RNeasy kit." Northern hybridization was with a 250-bp *Hin*dIII-*Pst*I fragment from the 5′ end of the mouse *Gapdh* gene, a 499-bp fragment comprising exon 7 of *Snrpn* (16), and *U2af1-rs1* probe 1 (10).

ChIP and PCR-SSCP—Histone extraction from cultured cells and analysis of purified histones on acetic acid/urea/Triton gels were according to Bonner *et al*. (21). Western blotting and immunostaining with antisera to acetylated histones were as described previously (22). Purification of nuclei, partial fractionation of chromatin with MNase to obtain fragments of predominantly 1–5 nucleosomes in length, and immunoprecipitation with affinity-purified antibodies were performed as described previously (23). The following antisera were used: R252/16 (to H4Ac16), R41/5 (to H4Ac5), R224/14 (to H3Ac14), and R47/9/18 (to H3Ac9/18) (24). For PCR-SSCP, 50 ng of each from the extracted DNA samples were used to PCR amplify (36 cycles; T-annealing = 60 °C) in the presence of [^{32}P]dCTP (1% of total dCTP) from two regions in *U2af1-rs1*: a 293-bp region of the 5′-UTR (forward, cgcagatcagacatactgcgg; reverse, tgtggtacggccagcctatg) and a 163-bp 3′-UTR region (forward, ctaattcccaaccaagttaca; reverse, aaaacaacatgggaagccag). *Snrpn* primers amplified a 228-bp region at the DMR1 (forward, agttgtgactgggatcctg; reverse, gcggcaacagaacttctacc). Denatured PCR products were resolved by SSCP gel electrophoresis (25). Following migration, gels were dried and exposed to x-ray films or analyzed by a phosphorimager (FLA3000, Fuji). The relative band intensities were calculated using the Quantity-One imaging software (Bio-Rad).

RESULTS

TSA Alters the Differential Acetylation of Maternal and Paternal U2af1-rs1 Alleles in ES Cells but Not in Fibroblasts—Acetylation studies were performed on interspecific hybrid cell lines. In all ChIP assays, the parental alleles were compared directly by using a combination of PCR amplification and electrophoretic detection of SSCP (25). For our studies, we selected a primary embryonic fibroblast line, EF1, that is (C57BL/6 × *Mus spretus*)F1 for proximal chromosome 11 on a homozygous C57BL/6 background (16) and a (C57BL/6 × *M. spretus*)F1 embryonic stem (ES) cell line (SF1-1; Ref. 20). In both cell lines, the two imprinted genes that we analyzed, *U2af1-rs1* and *Snrpn*, had maternal DNA methylation at their DMRs and were expressed exclusively from the paternal allele (16, 20).

To explore the role and regulation of the paternal allele-specific H3 and H4-lysine 5 acetylation at *U2af1-rs1* gene, we set out to alter levels of acetylation by treatment of the interspecific hybrid cells with TSA. To minimize pleiotropic or cell cycle effects of TSA (18), we restricted the treatment time to 6 h at a concentration of 300 nM. In initial experiments, we found that in undifferentiated SF1-1 ES cells, prolonged TSA treatment (12 or 24 h at 100 or 300 nM) led to extensive detachment of cells from the culture dish and severe restriction of cell growth after removal of the drug. In contrast, TSA treatment of SF1-1 ES cells (and EF1 fibroblasts) for only 6 h did not give rise to gross morphological changes or cell detachment, and cells continued to grow normally after TSA removal (data not shown). We analyzed global levels of H3 and H4 acetylation in untreated cells and in cells harvested immediately after the 6-h treatment. In the SF1-1 ES cells, the short treatment was sufficient to induce a major increase in histone acetylation, detected by Coomassie Blue staining and Western blotting of bulk histones separated on acetic acid/urea/Triton X-100 gels (Fig. 1). Gel scanning showed that ~80% of all histone H4 in the TSA-treated ES cells was present in the tetra-, tri-, or diacetylated forms, as compared with less than 5% before treatment. Based on immunostaining with antisera to H3 acetylated at either lysine 14 or lysines 9 and/or 18 (the antiserum used does not discriminate between H3 acetylated at lysines 9 and 18), TSA treatment also induces a dramatic increase in global levels of H3 acetylation (Fig. 1*A* and data not shown). Very similar results were obtained with the embryonic fibroblast line EF1 (Fig. 1*B*).

The effects of TSA on acetylation at *U2af1-rs1* were investigated by performing chromatin immunoprecipitation (ChIP) on untreated and TSA-treated SF1-1 and EF1 cells. Antibody-bound fractions were assayed for paternal and maternal DNA from the 5′- and 3′-UTRs of the *U2af1-rs1* gene, by PCR amplification and electrophoretic detection of SSCP (Fig. 2*A*). Of critical importance for the application of PCR-SSCP to allelic acetylation studies is the demonstration that PCR amplifications from (C57BL/6 × *M. spretus*)F1 genomic DNA give equal amounts of C57BL/6 and *M. spretus*-specific fragments on SSCP gels. This is shown in Fig. 2 for the *U2af1-rs1* regions analyzed.

We have shown previously that, in untreated ES cells, the methylated, maternal *U2af1-rs1* allele is underacetylated at

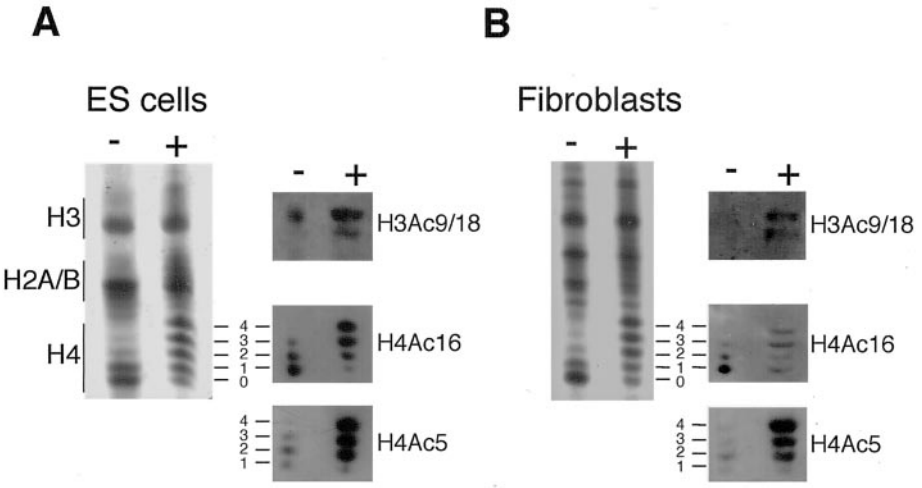

FIG. 1. **TSA increases global H3 and H4 acetylation in ES cells and fibroblasts.** Histones were extracted from untreated (−) and TSA-treated (+) SF1-1 ES cells (*A*) and EF1 embryonic fibroblasts (*B*) and resolved on acetic acid/urea/Triton X-100 gels. Subsequently, gels were stained with Coomassie Blue (*left panels*) or transferred to nylon filter and immunostained with antisera to H4Ac16, H4Ac5, and H3Ac9/18 (*right panels*). The migration of histones is as described by Bonner *et al.* (28) and was confirmed by immunostaining

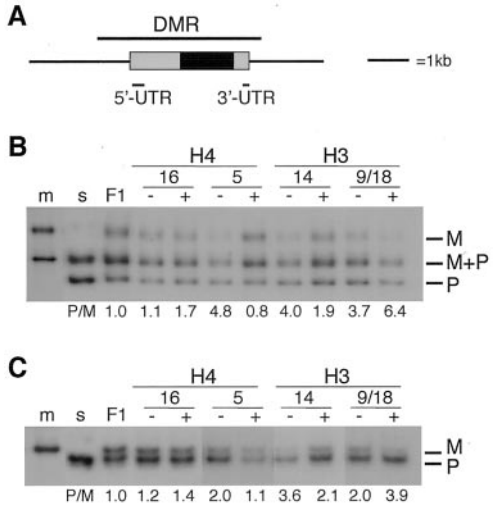

FIG. 2. **In ES cells, TSA alters acetylation of H4-lysine 5 and H3-lysine 14 acetylation along *U2af1-rs1*.** *A*, map of the *U2af1-rs1* gene, shown as a *box* with its coding part in *black*. The *line* above represents the domain of maternal DNA methylation and differential generalized DNase I sensitivity (10). *Small bars* indicate the regions analyzed by PCR-SSCP. *B*, acetylation at the 5′-UTR of *U2af1-rs1*. ChIP was performed simultaneously on nontreated (−) and TSA-treated (+) SF1-1 ES cells, and PCR on the corresponding DNA samples was with primers from the 5′-UTR. The *first three lanes* show PCR products (after SSCP electrophoresis) from control liver DNAs of C57BL/6 (*m*), *M. spretus* (*s*), and (C57BL/6 × *M. spretus*)F1 (*F1*), respectively. Subsequent lanes show PCR amplifications following ChIP with antisera to H4Ac16, H4Ac5, H3Ac14, and H3Ac9/18, respectively. Maternal (*M*) and paternal (*P*) allele-specific fragments are indicated. *C*, acetylation at the 3′-UTR of *U2af1-rs1*. Amplification from the same DNAs was with primers from the 3′-UTR.

TABLE I
Summary of PCR-SSCP data, presented as the ratios of paternal over maternal acetylation

	U2af1-rs1, 5′-UTR	*U2af1-rs1*, 3′-UTR	*Snrpn*, DMR1
SF1–1 ES cells			
H4:			
Ac16	1.1	1.2	2.0
Ac5	4.8	2.0	6.8
H3:			
Ac14	4.0	3.6	6.8
Ac9/18	3.7	2.0	>10
SF1–1 ES cells + TSA			
H4:			
Ac16	1.7	1.4	2.3
Ac5	0.8	1.1	4.0
H3:			
Ac14	1.9	2.1	6.3
Ac9/18	6.4	3.9	>10
EF1 fibroblasts			
H4:			
Ac16	1.0	1.0	
Ac5	2.4	2.1	
H3:			
Ac14	>10	>10	
Ac9/18	>10	>10	
EF1 fibroblasts + TSA			
H4:			
Ac16	1.3	0.9	
Ac5	2.2	2.0	
H3:			
Ac14	>10	>10	
Ac9/18	>10	>10	

H4 lysine 5 and at H3 lysines 14 and 9/18 but not at H4 lysines 8, 12, and 16 (16). In TSA-treated ES cells, ChIP/PCR-SSCP assays show that levels of histone H4 acetylated at lysine 5 (H4Ac5) became similar on the maternal and paternal *U2af1-rs1* alleles at both the 5′- and the 3′-UTR (Fig. 2, *B* and *C*, respectively). TSA also has an effect on H3Ac14 levels; paternal/maternal ratios are about 2-fold lower in TSA-treated cells, although the paternal allele remains more highly acetylated. In contrast, TSA did not effect the relatively low levels of H3Ac9/18 on the maternal *U2af1-rs1* allele. If anything, the measured ratios of paternal over maternal H3-K9/18 acetylation were even higher than in the untreated cells (Fig. 2, *B* and *C*). These findings, summarized in Table I, suggest that in ES cells, TSA induces a significant gain of maternal acetylation on H4-lysine 5 and H3-lysine 14. In contrast to the specific effects in ES cells, in the EF1 embryonic fibroblasts, the preferential acetylation of both H3 and H4 on the paternal *U2af1-rs1* allele remained essentially unaltered after growth for 6 h in the presence of TSA (Table I). Hence, despite its pronounced effects on global levels of H3 and H4 acetylation in these differentiated cells (Fig. 1*B*), TSA did not affect the relative allelic levels of acetylation along *U2af1-rs1*.

U2af1-rs1 Expression and DNA Methylation Are Unaltered in TSA-treated Cells—TSA treatment did not affect the expression of the *U2af1-rs1* gene. Levels of *U2af1-rs1* mRNA measured by Northern hybridization were unaltered by TSA treatment of ES cells and fibroblasts (Fig. 3*A*). When assayed by the more sensitive reverse transcriptase PCR amplification technique, expression in the TSA-treated ES cells continued to be from the paternal chromosome exclusively (Fig. 3*B*). Before treatment of the SF1-1 cells with TSA, all CpG methylation at the *U2af1-rs1* locus was present on maternal chromosomes. Specifically, about 85% of SF1-1 cells showed maternal methylation of a unique *Not*I restriction site in the 5′-UTR and of 24

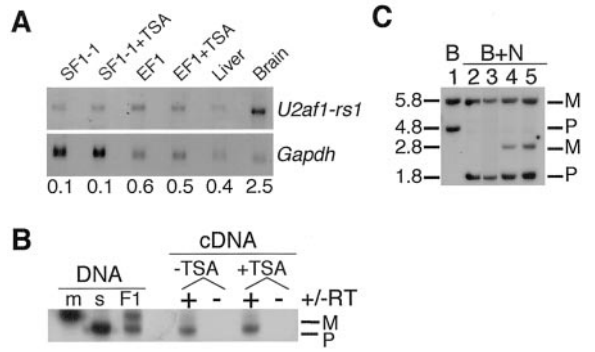

FIG. 3. **U2af1-rs1 expression and methylation are unaltered in TSA-treated cells.** *A*, Northern analysis of total RNA samples. Hybridization was with *U2af1-rs1* probe 1 (*upper panel*) and a *Gapdh* control probe, respectively. The latter yielded the same relative band intensities as a probe hybridizing to 18 S and 28 S RNA (data not shown). U2af1-rs1:Gapdh ratios of band intensities are indicated. *B*, unaltered paternal *U2af1-rs1* expression. The *lanes* to the *left* show amplifications from C57BL/6 (*m*), *M. spretus* (*s*), and (C57BL/6 × *M. spretus*)F1 (*F1*) DNAs, respectively. The *lanes* to the *right* show expression from cDNA samples corresponding to untreated and TSA-treated SF1-1 ES cells. To exclude possible DNA contamination, we performed parallel assays without the addition of reverse transcriptase (−). *C*, *U2af1-rs1* methylation in EF1 and SF1-1 cells. *Bgl*II (*B*) and *Bgl*II + *Not*I (*B*+*N*)-digested DNA samples were analyzed by Southern hybridization with probe 1. *Lanes 1* and *2*, (C57BL/6 × *M. spretus*)F1 liver; *lane 3*, EF1 fibroblasts; *lane 4*, SF1-1 ES cells; *lane 5*, TSA-treated SF1-1 cells. Maternal (*M*) and paternal (*P*) specific fragments are indicated; their sizes are given in kb. The intensity of the 2.8-kb band in *lanes 4* and *5* indicates that both in SF1-1 and in TSA-treated SF1-1 cells, ~15% of the maternal chromosomes are not methylated at the *Not*I restriction site. For the location of the *Not*I site and probe 1, see Fig. 4*A*.

*Hpa*II restriction sites distributed along the locus. This was not altered by TSA treatment, and *U2af1-rs1* methylation in EF1 fibroblasts was also unaltered by TSA (Fig. 3*C* and data not shown).

TSA Alters the Differential Sensitivity to DNase I of Maternal and Paternal U2af1-rs1 Chromatin in ES Cells but Not Fibroblasts—In adult brain and liver (10), and in kidney (Fig. 4*B*), chromatin along the repressed maternal *U2af1-rs1* allele is severalfold less sensitive to DNase I *in vivo* than its paternal counterpart. A similar differential was observed in the ES cells and the fibroblasts, at the passages that were analyzed in this study. Hence, when incubating nuclei purified from these cells at a range of increasing concentrations of DNase I, the repressed maternal allele became fully digested only at severalfold higher enzyme concentrations than the active paternal allele (Fig. 4, *C* and *D*). To investigate whether this allelic difference in generalized sensitivity along *U2af1-rs1* is associated with paternal allele-specific histone acetylation, we studied DNase I sensitivity in the TSA-treated SF1-1 ES cells. Using a Southern blotting approach, we found that the maternal *U2af1-rs1* copy invariably becomes more DNase I-sensitive upon TSA treatment, acquiring a generalized sensitivity to DNase I similar to that of the paternal chromosome. This was observed using a *Bgl*II + *Sac*I restriction fragment length polymorphism (RFLP) (Fig. 4*B*) that encompasses the 3′-half of the gene, in which no hypersensitive sites are present (10). In contrast, TSA did not have a predominant effect on the differential sensitivity in the EF1 fibroblasts, in which the maternal *U2af1-rs1* allele remained more resistant to DNase I than the paternal chromosome (Fig. 4*D*). This agrees with our finding of unaltered allelic acetylation in these TSA-treated differentiated cells.

To analyze nuclease sensitivity at the opposite end of the gene, we made use of the PCR-SSCP polymorphism at its 5′ extremity (see Fig. 2), encompassing 293 bp in which no hypersensitive sites are present (10). Hence, nuclei from control and TSA-treated SF1-1 and EF1 cells were incubated at increasing concentrations of DNase I, and extracted DNA samples were used to PCR amplify from the 5′-UTR, followed by migration of the PCR products through a nondenaturing polyacrylamide gel (precisely as for the PCR-SSCP analysis of immunoprecipitated chromatin, Fig. 2). This showed that in the untreated ES cells and fibroblasts, the paternal allele was more readily digested by DNase I than the maternal allele (Fig. 5). In the TSA-treated ES cells, however, similar amounts of maternal and paternal PCR products were amplified at all but the highest nuclease concentration used, indicating that the maternal and the paternal alleles had become much more similar in their sensitivity to DNase I. In the EF1 fibroblasts, TSA treatment did not change the differential PCR amplification at the 5′-UTR; the repressed maternal allele remained more resistant to DNase I digestion than the expressed paternal allele (Fig. 5).

The differential, generalized sensitivity to DNase I seems not to be associated with significant differences in the positioning of nucleosomes along the maternal and paternal *U2af1-rs1* alleles. This was apparent from analysis of mice that were maternally (Matdi11) or paternally (Patdi11) disomic for chromosome 11. Purified liver nuclei from Matdi11 and Patdi11 mice were incubated for increasing lengths of time with micrococcal nuclease. Genomic DNA was extracted from these MNase series, digested with the restriction enzyme *Hin*dIII, Southern blotted, and hybridized with small probes (probes 7 and 8, respectively) from the opposite extremities of a 4.2-kb *Hin*dIII fragment that comprises most of the *U2af1-rs1* gene (Fig. 6). The MNase digestion profiles revealed by hybridization with both these probes appeared identical for the Matdi11 and Patdi11 nuclei (Fig. 6). This finding agrees with our earlier observation (10) that the parental alleles of *U2af1-rs1* have a similar sensitivity *in vivo* to MNase. We also analyzed nuclei from early-passage androgenetic and parthenogenetic ES cells, and in these monoparental cells the *U2af1-rs1* MNase digestion profiles were similar (data not shown).

In adult tissues, the *U2af1-rs1* locus displays differential sensitivity to the restriction endonuclease *Msp*I, with chromatin on the silent and methylated maternal chromosome being highly resistant to this methylation-*insensitive* enzyme (10). This difference may be attributed to the presence of methyl-CpG-binding domain (MBD) proteins on the maternal allele (16). In contrast to its pronounced effects on the generalized DNase I sensitivity in ES cells, TSA did not significantly alter the differential *Msp*I sensitivity along the *U2af1-rs1* locus (data not shown).

At DMR1 of Snrpn, TSA Treatment Does Not Affect Allelic Differences in Histone Acetylation or DNA Methylation—The 5′ part of the imprinted *Snrpn* gene has a DMR (DMR1, Fig. 7*A*) at which methylation is established in the female germ line and is maintained in all embryonic lineages (26). In a previous study, we established that the DMR1 of *Snrpn* has paternal allele-specific patterns of histone acetylation. As for *U2af1-rs1*, the differential acetylation at histone H4 is most pronounced at lysine 5, whereas at histone H3, all lysine residues analyzed show paternal acetylation (Ref. 16; Fig. 7*B*).

We find that TSA causes no detectable change in the relative levels of acetylation on the maternal and paternal *Snrpn* alleles. With antibodies to acetylated H3 (lysines 14, 9, and 18) and H4 (lysine 5), most of the chromatin precipitated from the DMR1 originated from the paternal chromosome, as in the untreated cells (Fig. 7*B*). The expression of *Snrpn* appeared also unaltered by TSA treatment of ES cells (Fig. 7, *C* and *D*). We were unable to perform allelic acetylation studies on the EF1 fibroblasts, since these are homozygous C57BL/6 for chro-

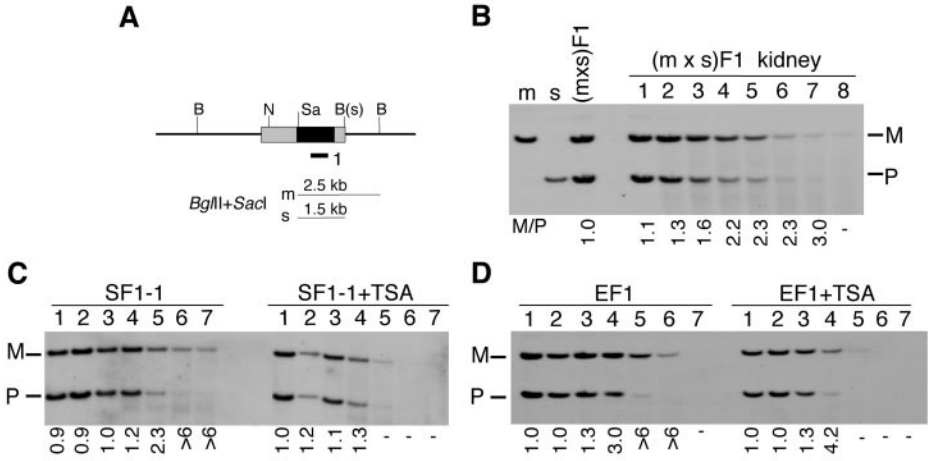

FIG. 4. **Southern-based analysis of DNase I sensitivity in TSA-treated cells.** *A*, map depicting the strategy used to analyze parental allele-specific DNase I sensitivity along *U2af1-rs1*. *Bgl*II (*B*), *Sac*I (*Sa*), and *Not*I (*N*) restriction sites and probe 1 are indicated. C57BL/6 (*m*)- and *M. spretus* (*s*)-specific *Bgl*II + *Sac*I fragments are shown underneath. We submitted the nucleotide sequence of this region to GenBank™ (accession number AF309654). *B*, DNase I assay on (C57BL/6 × M. spretus)F1 kidney cells. After purification, nuclei were incubated at increasing concentrations of DNase I (*lanes 1–8* correspond to 0, 50, 100, 200, 300, 400, 500, and 750 units of DNase I/ml, respectively). DNA was extracted subsequently and digested with *Bgl*II + *Sac*I, followed by Southern hybridization with probe 1. The 2.5-kb, *M. musculus*-specific (maternal, *M*) and the 1.5-kb, *M. spretus*-specific (paternal, *P*) bands are indicated. Measured maternal:paternal (*M*/*P*) ratios of band intensities are indicated underneath the lanes; −, bands are too weak to determine M/P ration. The *three lanes* to the *left* show *Bgl*II + *Sac*I-digested genomic DNAs from C57BL/6 (*m*), *M. spretus* (*s*), and (C57BL/6 × *M. spretus*)F1 (*F1*) DNAs, respectively. *C*, DNase I assay on ES cells. Nuclei from untreated and TSA-treated SF1-1 cells were incubated at increasing concentrations of DNase I (*lanes 1–7* correspond to 0, 50, 100, 200, 300, 400, and 500 units of DNase I/ml, respectively). Southern hybridization with probe 1 was the same as in *B*. Maternal:paternal ratios are indicated underneath the lanes. *D*, DNase I assay on EF1 fibroblasts. *Lanes 1–7* correspond to 0, 100, 200, 300, 400, 500, and 750 units/ml, respectively. Southern hybridization with probe 1 was the same as in *B*.

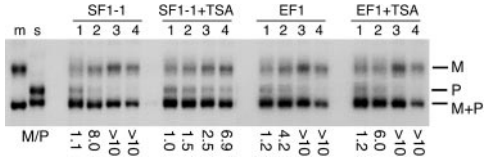

FIG. 5. **PCR-based analysis of DNase I sensitivity in TSA-treated cells.** Nuclei were purified from untreated and TSA-treated SF1-1 ES cells and EF1 fibroblasts and incubated with DNase I at increasing enzyme concentration (*lanes 1–4* correspond to 0, 300, 600, and 900 units/ml, respectively). Extracted DNA samples were used to PCR amplify with the primers from the 5′-UTR, followed by SSCP electrophoresis of the PCR products. Maternal (*M*) and paternal (*P*) specific bands are indicated, and ratios of maternal:paternal band intensities are indicated underneath the lanes. To the *left* are shown control amplifications from C57BL/6 (*m*) and *M. spretus* (*s*) genomic DNA, respectively,

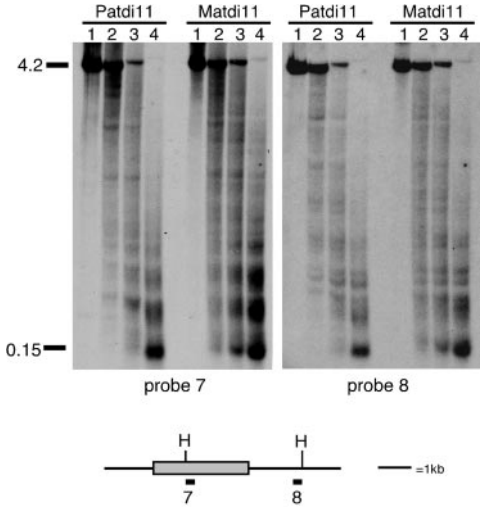

FIG. 6. **MNase digestion profiles in Matdi11 and Patdi11 mice.** Mice were analyzed that were paternally (*Patdi11*) or maternally (*Matdi11*) disomic for proximal chromosome 11. Liver nuclei were incubated with MNase for increasing periods of time (*lanes 1–4* correspond to 0, 30, 60, and 90 s, respectively). DNA samples were digested with *Hin*dIII and analyzed by Southern hybridization with probes 7 (*left panel*) and 8 (*right panel*), respectively. Hybridization with total genomic DNA established that the overall digestion by MNase was comparable in both the panels (data not shown). The lowest visible band is ~150 bp and corresponds to the mononucleosome. The map indicates the 4.2-kb *Hin*dIII (*H*) fragment relative to the *U2af1-rs1* gene (*gray box*) and probes 7 and 8 (*bars* underneath).

mosome 7 (where *Snrpn* resides). However, TSA did not lead to any detectable increase in *Snrpn* expression in these cells (Fig. 7*C*).

DISCUSSION

A key finding in this study is that whereas TSA induces global hyperacetylation on core histones H3 and H4, only partial (or no) effects are observed at the constitutive DMRs of the imprinted loci analyzed. Only in undifferentiated ES cells, at *U2af1-rs1* but not at *Snrpn*, did brief TSA treatment lead to a selective increase in the relative levels of histone acetylation on the repressed maternal chromosome. The TSA-induced changes in ES cells are confined to specific lysine residues and are associated with increased sensitivity to DNase I along the imprinted locus. These findings raise questions about the regulation of histone deacetylation at DMRs and its role in chromatin organization and gene repression.

Maintenance of Differential Histone Acetylation at the U2af1-rs1 and Snrpn DMRs—The observed effects of TSA on *U2af1-rs1* were cell type- and lysine residue-specific. In ES cells, TSA abolished the paternal/maternal difference in H4Ac5 at *U2af1-rs1* and reduced, but did not eliminate, the difference in H3Ac14. In contrast, there was no evidence for a gain in H3Ac9/18 on the maternal allele, despite the fact that TSA treatment led to a major increase in overall levels of H3-K9/18 acetylation in the ES cells. One interpretation of this result is that continuous HDAC activity is necessary to maintain the allelic acetylation differences in H4Ac5 and H3Ac14 in ES cells. In contrast, in primary embryonic fibroblast cells, TSA had no effect on the relative levels of H3Ac14, H3Ac9/18, or H4Ac5 on the maternal and paternal *U2af1-rs1* alleles. Although underlying mechanism(s) need to be determined, this difference be-

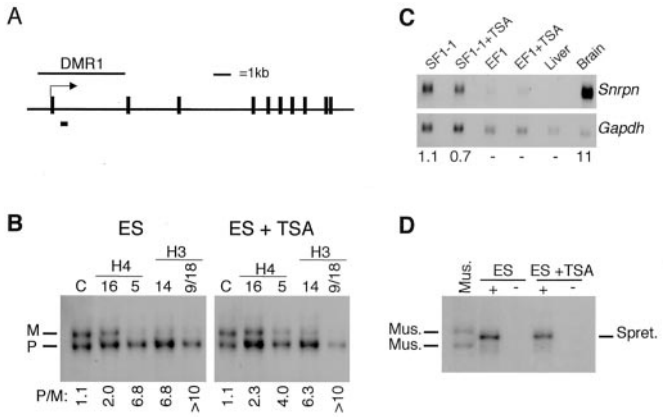

FIG. 7. **Allelic acetylation at DMR1 of *Snrpn* is unaltered by TSA.** *A*, map of the *Snrpn* gene, with exons 1–10 (*filled boxes*) and the differentially methylated region comprising exon 1 (DMR1, *horizontal bar*) as defined by Shemer *et al.* (26). The *small bar* below indicates the sequences analyzed by PCR-SSCP. *B*, acetylation of *Snrpn* in untreated (*ES*) and TSA-treated (*TSA*+*TSA*) ES cells. PCR-SSCP was performed on the same ChIP assays as described in the legend to Fig. 2*B*, and amplification was with primers from the DMR1 of *Snrpn*. *Lanes C* correspond to amplification from input chromatin without the addition of antiserum. *C*, *Snrpn* expression in TSA-treated ES cells. Northern blot hybridization was with an Snrpn, exon 7 (*upper panel*) and a *Gapdh* probe, respectively. Snrpn:Gapdh ratios of band intensities are indicated. This yielded the same relative intensities as compared with a probe hybridizing to 18 S and 28 S RNA (data not shown). *D*, unaltered paternal *Snrpn* expression. The *lane* to the *left* shows amplification (201-bp fragment covering exons 1–3) from C57BL/6 cDNA (from liver). To the *right*, amplification from cDNA samples corresponding to untreated (*ES*) and TSA-treated (*ES*+*TSA*) SF1-1 cells is shown; parallel assays were performed without the addition of reverse transcriptase (−). After denaturation, *Mus musculus* (*Mus.*)- and *M. spretus* (*Spret.*)-specific amplification products were separated by SSCP electrophoresis.

tween the two cell types would imply that acetylation patterns at *U2af1-rs1* become somehow stabilized and resistant to TSA upon differentiation.

In contrast to the results with *U2af1-rs1*, at the constitutive DMR of the *Snrpn* gene, we found no evidence for allelic changes in H3 and H4 acetylation in TSA-treated ES cells. One possible interpretation of this finding is that, in contrast to the regulation of global histone acetylation, which seems to involve continuous HDAC activity, there is a strongly reduced, or possibly cell cycle-regulated, turnover of histone acetate groups at these two DMRs. Alternatively, the HDAC activities that maintain the allelic acetylation patterns at *U2af1-rs1* and *Snrpn* could differ in their sensitivities to TSA, either because the enzymes involved are distinct or because they are associated with different proteins that alter their catalytic properties. For example, the NAD-dependent deacetylase SIR2 is highly resistant to TSA (27). The selective effect of TSA on H4-lysine 5 and H3-lysine 14 acetylation at *U2af1-rs1* raises the possibility that the enzyme(s) involved are specific for these lysine residues. Several HDACs show preferences for specific histones or lysine residues. Histone deacetylase Hda1p in yeast, for instance, preferentially deacetylates H3 *in vitro* (28), while histone acetylation patterns in mutants lacking this activity suggest a preference for H3 and H2B *in vivo* (29). Yeast HOS3p has a preference for H4Ac5 and H4Ac8 and H3Ac14 and H3Ac23 (30). Specificity can also be substrate-dependent. HDAC1, as part of the NuRD complex, for example, deacetylates all H4 lysines in free histones, but only lysines 5, 8, and 12 in chromatin (31). To our knowledge, so far no HDACs have been shown to be specific for H4Ac5 or H3Ac14 in chromatin.

Histone Acetylation, Chromatin Conformation, and DNA Methylation—In ES cells, TSA abolished paternal/maternal differences in generalized DNase I sensitivity along *U2af1-rs1*, while at the same time, allelic differences in H4Ac5 and H3Ac14 were reduced or abolished. In differentiated embryo fibroblasts, in contrast, there was no detectable change in relative levels of H3-K14 or H4-K5 acetylation and no allele-specific increase in DNase I sensitivity. These correlations do not, in themselves, establish H4-lysine 5 and/or H3-lysine 14 as mediators of DNase I sensitivity. They do, however, demonstrate that DNase I sensitivity on the maternal *U2af1-rs1* allele is not dependent on global changes in histone acetylation but may be regulated by the selective acetylation/deacetylation of specific lysine residues on H3 and H4. Our study did not consider core histones H2A and H2B, and we do not exclude a possible co-involvement of acetylation at these core histones. Now that suitable antisera are becoming available, this can be investigated. It was reported recently that chromatin at silenced transgenes acquires increased DNase I sensitivity *in vivo* after only a few hours of TSA treatment (32), and a correlation between histone underacetylation and chromatin compaction has also been demonstrated at the chicken β-globin chromosomal domain (33). It is unclear how precisely deacetylation of lysine residues on H3 and H4 leads to compaction of chromatin. The N-terminal tail of histone H4 links neighboring nucleosomes in core particle crystals, and such interactions might influence chromatin compaction *in vivo* (34). Indeed, it has been established that the N terminus of H3 is essential for the formation of condensed chromatin fibers (35), and several recent *in vitro* studies show that the extent of histone deacetylation at tail domains influences chromatin condensation (36, 37). Alternatively, acetylation of specific residues on H3 and/or H4 could prevent, either directly or indirectly, the association of nonhistone proteins that are involved in chromatin compaction. Centromeric chromatin is particularly susceptible to the effects of TSA and loses its ability to retain heterochromatin protein-1 (HP1) on prolonged treatment with this HDAC inhibitor (38).

TSA treatment did not induce methylation changes at *U2af1-rs1*, and the chromatin on the methylated maternal chromosome remained highly resistant to the restriction endonuclease *Msp*I (which recognizes sites that can be methylated but is not methylation-sensitive). One possible interpretation of the unaltered *Msp*I resistance of chromatin is that there is continued binding of proteins to methylated CpG dinucleotides. Several studies have demonstrated physical association between HDACs and MBD proteins (reviewed in Ref. 6), and in a previous study we demonstrated *in vivo* association of MECP2 with the methylated maternal *U2af1-rs1* allele (16). Such association of specific MBD proteins to methylated DNA represents an attractive targeting mechanism that could, at least partially, account for the observed low acetylation at histones on the maternal *U2af1-rs1* allele.

We found no evidence that TSA induces expression of *U2af1-rs1* or *Snrpn*. A few hours of incubation with TSA also failed to de-repress the silent parental alleles of the imprinted *Igf2* and *H19* genes on mouse chromosome 7 (39). In contrast, prolonged treatments of cultured cells with TSA has been reported to induce expression of the normally silent allele of the imprinted *Igf2* gene and the *Igf2*-receptor gene on mouse chromosome 17 (17, 40, 41). In these experiments, cells were grown for 24 h in the presence of the HDAC inhibitor. Perhaps, passage through S phase, or even a complete cell cycle, is necessary before the switch to a new transcriptional state can be accomplished at imprinted genes. We note, however, that TSA treatments of up to 72 h do not lead to de-repression of the silent maternal alleles of the *U2af1-rs1* and *Snrpn* genes (15, 41), and this supports our main finding that the DMRs at these imprinted

loci are particularly resistant to the effects of this HDAC inhibitor.

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