DNA Methylation Reactivates a Subset of Imprinted Genes in Uniparental Mouse Embryonic Fibroblasts

Aboubaker El Kharrouri, Graziella Piras, and Colin L. Stewart
From the Cancer and Developmental Biology Laboratory, Division of Basic Sciences, NCI-FCRDC, National Institutes of Health, Frederick, Maryland 21702

Although most imprinted genes show allelic differences in DNA methylation, it is not clear whether methylation regulates the expression of some or all imprinted genes in somatic cells. To examine the mechanisms of silencing of imprinted alleles, we generated novel uniparental mouse embryonic fibroblasts exclusively containing either the paternal or the maternal genome. These fibroblasts retain parent-of-origin allele-specific expression of 12 imprinted genes examined for more than 30 cell generations. We show that p57Kip2 (cyclin-dependent kinase inhibitor protein 2) and Igf2 (insulin-like growth factor 2) are induced by inhibiting histone deacetylases; however, their activated state is reversed quickly by withdrawal of trichostatin A. In contrast, DNA demethylation results in the heritable expression of a subset of imprinted genes including H19 (H19 fetal liver mRNA), p57Kip2, Peg3/Plce1 (paternally expressed gene 3), and Zac1 (zinc finger-binding protein regulating apoptosis and cell cycle arrest). Other imprinted genes such as Grb10 (growth factor receptor-bound protein 10), Pegl/Mest (paternally expressed gene 1/mesoderm-specific transcript), Sgc6 (epsilon-sarcoglycan), Snrnp (small nuclear ribonucleoprotein polypeptide N), and U2af1 (U2 small nuclear ribonucleoprotein auxiliary factor), remain inactive, despite their exposure to inhibitors of histone deacetylases and DNA methylation. These results demonstrate that changes in DNA methylation but not histone acetylation create a heritable epigenetic state at some imprinted loci in somatic cells.

Normal development of mammalian embryos requires the genetic contribution of both maternal and paternal genomes (1, 2). Uniparental mouse embryos, in which the entire genome is either of maternal (parthenotes) or paternal (androgenotes) origin, usually die during early stages of embryogenesis (2, 3). Lethality of uniparental embryos appears to be caused by either the lack of, or overexpression of, specific genes that are imprinted and only expressed from the nonimprinted parental allele. More than 35 autosomal genes in the mouse exhibit parent-of-origin, allele-specific imprinting in embryonic and adult tissues. The imprint, once set, is stable during mitosis but is reversed and reset by passage through meiosis during gametogenesis (4, 5). Given the epigenetic nature of imprinting, much attention has centered on whether DNA methylation is crucial to the establishment and maintenance of the silent imprinted allele (6, 7). Most imprinted genes show parental differences in methylation patterns (7–10), although the extent varies among the different genes. Furthermore, despite changes in global levels of DNA methylation in early embryogenesis, methylation of some imprinted genes remains constant even at the blastocyst stage, whereas the rest of the genome is hypomethylated (8, 11, 12). After implantation, most of the CpG sequences are progressively methylated, except those located in the promoter region of active “housekeeping” genes (13). These findings together with the derivation of embryos deficient in methyltransferases, in particular DNA methyltransferase 1, suggest that proper expression of some imprinted genes (10, 14) requires DNA methylation. However, it is unclear whether methylation is involved in the regulation of some or all imprinted alleles. If methylation regulates the monoallelic expression of imprinted genes, demethylation should reactivate silent alleles in vitro. We tested this hypothesis by analyzing the reactivation of imprinted alleles in a series of novel nonexpressing uniparental mouse embryonic fibroblasts (MEFs)1 treated by demethylating agents 5-azacytidine (AzaC) or 5-aza-2′-deoxycytidine (AzadC) alone or in combination with a histone deacetylase inhibitor, trichostatin A (TSA). Our results suggest that both DNA methylation and histone deacetylase activities regulate the differential allelic expression of some but not all imprinted genes in somatic cells and that the epigenetic modifications required for maintenance of monoallelic expression vary among different imprinted loci. Significantly, the activated state induced by DNA demethylation, but not by histone acetylation, is propagated and stably inherited during mitosis, indicating that methylation is required for long term repression of some imprinted genes.

MATERIALS AND METHODS

Derivation of Wild-type (WT) and Uniparental MEFs—WT and uniparental MEFs were derived from explanted day 15 (day of plug = day 1) embryos after removing the head and internal organs. Androgenetic (AG) MEFs were generated from chimeras made by injecting androgenetic ES cells, constitutively expressing the Neo gene (15, 16), into blastocysts. Parthenogenetic (PG) MEFs were generated from PG 171 WT chimeric embryos. PG embryos were derived by ethanol activation of eggs, with suppression of polar body formation, and were then aggregated at the 4- or 8-cell stage with WT embryos (17). The PG eggs were derived from mouse lines that constitutively expressed the Neo gene (15). The AG and PG MEFs were derived by culturing the primary explants in media supplemented with G418 for at least 1 week which selected against the WT cells lacking the NeoR gene.

1 The costs of publication of this article were defrayed in part by the page charge. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2 To whom correspondence should be addressed: CDBL, NCI-FCRDC, Bldg. 539, Rm. 135, 1050 Boyles St., P. O. Box B, Frederick, MD 21702. Tel.: 301-846-5158; Fax: 301-846-7117; E-mail: elkharroubia@mail.ncifcrf.gov.

3 Present address: Life Technologies Inc, Rockville, MD 20849.

This paper is available on line at http://www.jbc.org
Ref. 21 and 25.

containing 0.3 M of RNA was prepared and analyzed by RT-PCR. For the
in a 20-

residual genomic DNA. 1.5 M of RNA was converted to cDNA, and quantitative PCR was performed using
serial dilutions to assure a linear amplification of the target and control
was converted to cDNA, and quantitative PCR was performed using
94 °C for 30 s, and 72 °C for 60 s performed in a PerkinElmer Life Sciences
cals). Amplification consisted of 25 or 30 cycles of 94 °C for 30 s, 60 °C
of cDNA in 25–50 μl

for 24 h and total RNA was prepared and analyzed by RT-PCR. For the

for 30 s, and 72 °C for 60 s performed in a PerkinElmer Life Sciences

for 30 s, and 72 °C for 60 s performed in a PerkinElmer Life Sciences

were digested with RNases A and T1 and subsequently analyzed by
polyacrylamide gel and visualized by autoradiography.
The relative intensities of the target and control mRNAs were quantified by phosphorimaging.

Western Blotting of Acetylated Histones H3 and H4—Uniparental MEFs were cultured for 24 h in the absence or presence of TSA (0.2, 1, or 5 μM) were harvested and nuclei prepared as described previously (19).
Nuclei (3–5 × 10^7) were resuspended in lysis buffer (10 mM HEPES, pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol, 1 mM AEBSF (ICN) and proteinase inhibitors complete (Roche)), then SDS was added to final concentration of 1%. 20 μg of proteins from treated and untreated AG and PG MEFs were separated by 16.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Hyper-acetylated histones were detected by anti-acetylated H3 and H4 antibodies (Upstate Biotechnology) and were visualized by chemiluminescence (Amersham Pharmacia Biotech). As control for the amount of protein loading, a parallel gel was stained with Coomassie Blue.

DNA Extraction and Methylation Assay—Genomic DNA was ex-
tracted by proteinase KSSD digestion, phenolchloroform extraction and isopropyl alcohol precipitation. For p57Kip2 (cyclin-dependent ki-
nase inhibitor protein 2) analysis, DNA was first digested with HindIII and XbaI followed by incubation with either HpaII orMspI for at least 16 h at 37 °C. For the methylation analysis of U2af1 (U2 small nuclear ribonucleoprotein auxiliary factor) and Srrpn (small nuclear ribonucleo-

pectoprotein polypeptide N) genes, DNA was digested first withBamHI and HindIII followed by HpaII orMspI. Digested DNA was analyzed by Southern blotting as described previously (19).

RESULTS

Expression of Imprinted Genes in Uniparental MEFs—We generated uniparental diploid MEFs containing exclusively either the paternal AG or the maternal PG genome. In these uniparental cells, the epigenetic regulation of both alleles should be identical, with an imprinted gene being either ex-
pressed or silent depending on the parent-of-origin profile of imprinting. Probes to a panel of imprinted genes showed that these cell lines stably retain parent-of-origin allele-specific imprinting status over 30 cell generations (Fig. 1). The paternally expressed genes, Igf2 (insulin-like growth factor 2), Peg1/Mest (paternally expressed gene 1/ mesoderm-specific transcript), Peg3/Pw1 (paternally expressed gene 3), Srrpn, and U2af1 (20–24) as well as the two newly identified imprinted genes Sgce (epsilon-sarcoglycan), and Zac1 (zinc finger-binding pro-

tein regulating apoptosis and cycle cell arrest) (25) were detected only in AG and WT MEFs. In contrast, the transcripts of maternally expressed H19 (H19 fetal liver mRNA), Grb10/Meg1 (growth factor receptor-bond protein 10/maternally expressed gene 1), and p57Kip2 (26–28) were detected exclusively in PG MEFs and WT MEFs. The one exception was the Igf2r1/M6Pr (insulin-like growth factor 2 receptor/mannose 6-phos-
phate receptor) (29). This gene is strongly expressed from the maternal allele (in PG and WT MEFs), but low levels of expression were also detected in the AG MEFs, indicating that the silencing of the paternal allele was not fully acquired in day 13 uniparental embryonic tissues. Furthermore, the tissue-specific imprinted genes Rasgfr1 (Ras protein-specific guanine nucleotide-releasing factor 1), which is expressed exclusively from the maternal allele in the brain, heart, and stomach (30),Pin1/Mash2 (paternally expressed gene 2), a tropho-
blast-specific maternal expressed gene (31), were not detected in WT and uniparental MEFs. The housekeeping genes, Gapd and Hprt, were expressed in all MEFs. These results demonstrate that AG and PG MEFs retain the correct expression pattern of 12 imprinted genes and provide unique and significant advantages to analyze mechanisms leading to allele-specific silencing of these genes.

Table 1

| Gene      | Primers*          | Amplified product |
|-----------|-------------------|-------------------|
| Grb10     | 5′-CATGAGATCGTGGTCCAAGTTG | 353              |
|           | 5′-ATCGCTATCTCCTGCACTGAGCC |                |
| H19       | 5′-CCACCTACATCCGCCTGATGTCG | 192              |
|           | 5′-GAGCTGCCCTGTCGATGTCG |                |
| Mash2     | 5′-CCCGCGAAGCTCTGAGTATGTCG | 308              |
|           | 5′-GACCTGCTCTCGGCGAGT |                |
| p57Kip2   | 5′-CTGAGGTTAATGTTGATGTCG | 236              |
|           | 5′-ACCTTGGAGCCGACCGTACCTG |                |
| Igf2      | 5′-CTGAGGTTAATGTTGATGTCG | 519              |
|           | 5′-GATGACCGACCGACCGATG |                |
| Peg1      | 5′-CTGACCGACCGACCGATG | 319              |
|           | 5′-GTCACCCCTACAGATGATGTCG |                |
| Peg3      | 5′-ATGATCATCAGACGAGGACACCC | 303              |
|           | 5′-CTCGGCCTCGTCGATGTCG |                |
| Rasgrf1   | 5′-TGGAGGACCCTCGGCTCAGACAG | 343              |
|           | 5′-TGGAGGACCCTCGGCTCAGACAG |                |
| Sgce      | 5′-GGGCGGCGAGGTGCGCGCTCCTC | 404              |
|           | 5′-GGCGACACATGATATAGACGGAG |                |
| Srrpn     | 5′-CTGCTGCTCTGCTGCTACTGTAGC | 356              |
|           | 5′-GACCTAGGGGCAATGCGCGGAC |                |
| U2af1     | 5′-CGGCTGACTACAGATAACCGGAG | 250              |
|           | 5′-CGAATGACATGCTGCCCATG |                |
| Zac1      | 5′-ATCTGGTCTCTACATGATGCTG | 465              |
|           | 5′-CTGCTGCTCTGCAATGCTG |                |
| Gapd      | 5′-ACGCCAGCTGCTGCTGCTG | 452              |
|           | 5′-TCCACCCACTGTGCTGTA |                |
| Hprt      | 5′-GCCGCGCCCGAGCGCCGCGGTG | 461              |
|           | 5′-GCTGCTACTGCTTAACCAGGGAAG |                |

* Primers for H19, Igf2, Sgce, and Zac1 were originally described in Refs. 21 and 25.

Treatement of Cells with Inhibitors of DNA Methylation and Histone Deacetylases—Uniparental MEFs were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and Pen/Strep. The day before treatment, cells were split to 50–60% density. For the experiment shown in Fig. 2, cells were treated with various concentrations of TSA (Wako) (0.2–5 μM) or AzaC or AzadC (Sigma) (1–10 μM) for 24 h and total RNA was prepared and analyzed by RT-PCR. For the experiment shown in Figs. 3 and 4, cells were incubated in media containing 0.3 μM of TSA for 72 h. For treatment with the AzadC or AzaC (Sigma), cells were incubated with medium containing initially 1 μM AzaC or AzadC for 24 h followed by 0.3 μM AzaC or AzadC for an additional 48 h. For the combined treatment with AzaC/TSA or AzadC/ TSA, cells were incubated with AzaC or AzadC at the final concentra-
tion of 1 μM for 24 h followed by 0.3 μM for another 24 h, after which 0.4 μM TSA was added in the presence of 0.3 μM AzaC or AzadC for an additional 24 h. After treatment, cells were either used to prepare total RNA or cultured in the absence of the drugs for up to 2 weeks.

RT-PCR Analysis of Imprinted Gene Expression—Total RNA was extracted from WT and uniparental MEFs using the RNAeasy kit (Qiagen) and treated with RNase-free DNase I (Promega) to eliminate residual genomic DNA. 1.5 μg of RNA was converted to cDNA using random primers and avian myeloblastosis virus reverse transcriptase in a 20-μl reaction for 60 min at 42 °C. PCR was performed with 1–2 μl of cDNA in 25–50 μl using Taq polymerase (Roche Molecular Biochemi-
cals). Amplification consisted of 25 or 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s performed in a PerkinElmer Life Sciences GeneAmp PCR machine 9600. Primers for all target genes are listed in Table 1.

For quantitative evaluation of the expression of imprinted genes in ununtreated, TSA- and/or AzadC-treated AG and PG MEFs, total RNA was converted to cDNA, and quantitative PCR was performed using serial dilutions to assure a linear amplification of the target and control genes (18). The levels of target gene expression were measured relative to the housekeeping genes Hprt (hypoxanthine phosphoribosyltransferase) and Gapd (glyceraldehyde-3-phosphate dehydrogenase) as internal controls. The amplified products were analyzed by Southern blot-
Reactivation of Imprinted Genes in Uniparental MEFs—To examine the roles that DNA methylation and histone deacetylation play in differential expression of imprinted genes, uniparental AG and PG MEFs were treated with increasing concentrations of either a specific inhibitor of histone deacetylase (32), TSA (0.2–5 μM, Fig. 2) or with inhibitors of DNA methylation, AzaC/AzadC (1–10 μM) for 24 h. RT-PCR was performed to determine whether imprinted genes were induced by drug treatment in nonexpressing cells. TSA treatment resulted in a dose-dependent induction of Igf2 and p57Kip2 in PG and AG MEFs, respectively (Fig. 2A). A modest induction of both Igf2 and p57Kip2 was observed in cells treated with a low dose of TSA (0.2 μM). TSA reactivation was significantly higher at 1 μM and decreased at 5 μM, possibly because of its adverse effect on cell proliferation and viability (33). Unlike Igf2 and p57Kip2, all of the other imprinted genes listed in Fig. 1 remained silent, despite induced accumulation of hyperacetylated histones H3 and H4 after culturing uniparental cells in the presence of different doses of TSA (Fig. 2B). Acetylation of core histones, in particular H3 and H4, is believed to play a role in chromatin unfolding and transcription regulation, as actively transcribed genes are enriched in hyperacetylated histones H3 and H4 (33–35). Western blot analysis showed that levels of acetylated histones H3 and H4 were very low (detectable only at high exposure, data not shown) in untreated AG and PG MEFs. Incubation with 0.2–5 μM TSA resulted in the accumulation of acetylated H3 and H4 as determined by antibodies directed against acetylated histones (Fig. 2B; 0.2, 1, and 5 μM TSA). Under these experimental conditions, among the imprinted genes analyzed (Fig. 1), only Igf2 and p57Kip2 were reactivated. At this point we cannot rule out whether the induction of these two genes is triggered by a locus-specific histone acetylation or by a secondary effect of a global histone acetylation.

In contrast, treatment with demethylating agents AzaC or AzadC for 24 h was not sufficient to induce detectable levels of imprinted gene mRNA in nonexpressing cells (data not shown). However, treatment with high doses of AzaC and AzadC (5–10 μM) for over 24 h was toxic and inhibited cell proliferation. To minimize the adverse effects of drug treatment on cell proliferation, a protocol was elaborated in which uniparental AG and PG MEFs were treated for 3 days with low dose of AzaC, AzadC, and/or TSA (see “Materials and Methods”). As shown in Fig. 3, this resulted in the activation of a subset of imprinted genes. Peg3 and Zac1 were reactivated in PG MEFs and, p57Kip2 and H19 in AG cells after exposure of cells to either AzaC or AzadC. Igf2 was induced in PG MEFs treated with TSA alone and was not responsive to demethylating agents, whereas both TSA and AzaC/AzadC synergistically activated p57Kip2 in AG MEFs. In contrast, Grb10, Peg1, Rasgrf1, Sgce, Snrpn, and U2af1 remained inactive after both treatments. The effects of TSA and AzadC on the expression of Igf2r were not assessed because the gene is not fully silenced in AG MEFs.

The levels of expression of a gene paternally imprinted, p57Kip2, and two maternally imprinted genes, Peg3 and U2af1, were evaluated further by RNase protection assay and/or quantitative RT-PCR (Fig. 4). Measurement by phosphorimaging analysis of changes in p57Kip2 mRNA levels revealed a significant induction of p57Kip2 expression after treatment with either TSA or AzadC (Fig. 4A). However, the combined treatment with AzadC and TSA synergistically activated p57Kip2 expression to about 5-fold higher levels than treatment with either AzadC or TSA alone. These results suggest that both histone deacetylase and DNA methylation activities mediate silencing of the paternal allele of p57Kip2 in cultured MEFs. Unlike p57Kip2, Peg3 was activated in PG MEFs treated with AzadC alone, whereas the addition of TSA antagonized the induction by AzadC in a combined treatment (Fig. 4B), demonstrating that in this case the hyperacetylation of histones has a negative effect on gene expression. Under the same protocols, treatment

Reactivation of Imprinted Genes in Nonexpressing Cells—To examine the roles that DNA methylation and histone deacetylation play in differential expression of imprinted genes, uniparental AG and PG MEFs were treated with increasing concentrations of either a specific inhibitor of histone deacetylase (32), TSA (0.2–5 μM, Fig. 2) or with inhibitors of DNA methylation, AzaC/AzadC (1–10 μM) for 24 h. RT-PCR was performed to determine whether imprinted genes were induced by drug treatment in nonexpressing cells. TSA treatment resulted in a dose-dependent induction of Igf2 and p57Kip2 in PG and AG MEFs, respectively (Fig. 2A). A modest induction of both Igf2 and p57Kip2 was observed in cells treated with a low dose of TSA (0.2 μM). TSA reactivation was significantly higher at 1 μM and decreased at 5 μM, possibly because of its adverse effect on cell proliferation and viability (33). Unlike Igf2 and p57Kip2, all of the other imprinted genes listed in Fig. 1 remained silent, despite induced accumulation of hyperacetylated histones H3 and H4 after culturing uniparental cells in the presence of different doses of TSA (Fig. 2B). Acetylation of core histones, in particular H3 and H4, is believed to play a role in chromatin unfolding and transcription regulation, as actively transcribed genes are enriched in hyperacetylated histones H3 and H4 (33–35). Western blot analysis showed that levels of acetylated histones H3 and H4 were very low (detectable only at high exposure, data not shown) in untreated AG and PG MEFs. Incubation with 0.2–5 μM TSA resulted in the accumulation of acetylated H3 and H4 as determined by antibodies directed against acetylated histones (Fig. 2B; 0.2, 1, and 5 μM TSA). Under these experimental conditions, among the imprinted genes analyzed (Fig. 1), only Igf2 and p57Kip2 were reactivated. At this point we cannot rule out whether the induction of these two genes is triggered by a locus-specific histone acetylation or by a secondary effect of a global histone acetylation.
Reactivation of Imprinted Genes in Uniparental MEFs

of PG MEFs with AzadC and/or TSA did not significantly change the expression levels of U2af1, Hprt, or Gapd (Fig. 4, B and C). Overall the results shown in Figs. 2–4 suggest that only a subset of imprinted genes is activated in response to inhibitors of histone deacetylation and/or DNA methylation.

AzaC and AzadC Treatments Correlate with DNA Demethylation—We analyzed the methylation status of p57Kip2 (induced by TSA and AzadC) and U2af1 (not responsive to either TSA or AzadC treatment) by comparing the digestion of genomic DNA from untreated, TSA-, AzaC-, or AzadC-treated AG and PG MEFs (Fig. 5). After treatment, cells were cultured in the absence of drugs for 3–7 days, and DNA was purified and digested with the methylation-sensitive HpaII or insensitive MspI enzymes and analyzed by Southern blotting. Blots were hybridized with probes derived from the promoter region of p57Kip2 (Fig. 5A) and U2af1 (Fig. 5B). DNA from WT MEFs, which carry both active and silent alleles of each imprinted gene, was used as a control.

In all samples, DNA from nonexpressing cells (AG for p57Kip2 and PG for U2af1), either untreated or TSA-treated, was relatively resistant to HpaII digestion, but was cut by MspI to produce heterogeneous fragments of various sizes (Fig. 5, A and B). The results demonstrated that in nonexpressing cells, p57Kip2 (in AG MEFs) and U2af1 (in PG MEFs) genes are densely methylated, whereas in the expressing cells DNA is unmethylated, as it is digested to the same extent with either HpaII or MspI (compare Fig. 5A, lanes 6 and 7, with Fig. 5B, lanes 7 and 9). After treatment with AzadC, p57Kip2 DNA from AG MEFs was readily digested with HpaII (Fig. 5A, compare lanes 2 and 4), demonstrating that AzadC induced extensive demethylation at this locus, particularly with regard to the appearance of a major band at 0.4 kilobase. However, DNA from the same cells treated with TSA (which also activated p57Kip2) showed only minor changes in HpaII digestion, indicating that TSA did not cause significant demethylation as seen in AzadC-treated samples. Similar results were obtained when blots were hybridized with U2af1 (Fig. 5B). DNA from AzaC- or AzadC-treated PG cells was partially digested with HpaII, whereas DNA from untreated cells was fully resistant to digestion (compare lanes 2–5). Thus, under these experimental conditions DNA is significantly demethylated after AzaC or AzadC treatment, whereas the same sequences in untreated cells are densely methylated. However, despite significant levels of DNA demethylation, U2af1 and other imprinted genes remained silent after treatment (Figs. 3 and 4), suggesting that partial demethylation was not sufficient to reactivate these silent alleles.

Transient Inhibition of DNA Methylation, but Not Histone Deacetylase, Promotes a Heritable State of Gene Expression—In mammals, propagation of the methylation state within CpG dinucleotides by the maintenance DNA methyltransferase occurs during or shortly after replication (36). Inhibition of DNA methylation by AzaC or AzadC leads to the reactivation of some imprinted genes, specifically H19, Peg3, p57Kip2, and Zac1,
whereas TSA treatment induces two genes, p57Kip2 and Igf2 (Fig. 3). To determine whether these induced patterns of expression are maintained after multiple cell divisions, uniparental MEFs initially treated for 3 days with TSA, AzaC, or AzadC (Fig. 6, lanes P1) were cultured for 7 days (lanes P2) or 15 days (lanes P4) after withdrawal of the inducing agents. The results shown in Fig. 6 revealed that the expression of H19, Peg3, p57Kip2, and Zac1 induced by transient treatment with either AzaC or AzadC is enriched and maintained over the course of multiple cell divisions in the absence of either inhibitor (compare P1 with P4). However, the TSA-induced transient expression of p57Kip2 and Igf2 was quickly reversed to a silent state after two to four cell cycles after drug withdrawal. These results demonstrate that changes in DNA methylation, but not levels of histone acetylation, create a dominant and heritable epigenetic state of gene expression at some imprinted loci.

**DISCUSSION**

In mammals, genomic imprinting results from the differential epigenetic modification to a subset of genes in the germ line, leading to parent-of-origin monoallelic expression during embryogenesis and in adult tissues. Studying the molecular basis that distinguishes the paternal and maternal alleles of imprinted genes has been complicated by the simple fact that all somatic tissues contain both maternal and paternal genomes. This necessitated the use of a variety of techniques to distinguish between the parental alleles, such as polymorphisms generated by intercrosses or the introduction of mutations or deletions into different imprinted genes. Here we have greatly simplified the analysis of imprinted gene expression by generating novel uniparental mouse fibroblast lines retaining parental specific expression is stably inherited during multiple cell generations in vitro, demonstrating that these uniparental cells provide unique advantages for analyzing the mechanisms of silencing of imprinted alleles. Treatment of uniparental cells

**FIG. 5.** DNA is demethylated in AG and PG MEFs treated with AzaC or AzadC. A, analysis of DNA methylation of the p57Kip2 gene in AG, PG, and WT MEFs. Genomic DNA from untreated (-) or treated (+) with AzadC or TSA was isolated and digested with HpaII (lanes 2–4, 7, 8, and 10) or MspI (lanes 5, 6, and 11) or left undigested (u, lanes 1 and 9) as indicated on the top of the panel. All DNAs in panel A were digested with HindIII/XbaI and analyzed by Southern blotting using a probe that hybridizes to the promoter region of p57Kip2. In the lower panel, the genomic structure of the mouse p57Kip2 is shown. The closed boxes numbered E1–E4 are exons 1–4. The horizontal arrow indicates the putative transcription start site. The positions of CpG islands and HpaII sites are indicated. B, evaluation of DNA methylation of U2af1 gene after treatment with AzaC or AzadC. Genomic DNA from untreated (-) or treated (+) MEFs was either undigested (u) or digested with HpaII or MspI as indicated on the top of the panel. DNA samples were also digested with HindIII/ BamHI, and the blot was hybridized with a probe spanning the U2af1 promoter region. In the lower panel, the genomic structure of the mouse U2af1, the positions of CpG islands and HpaII sites are indicated.

**FIG. 6.** Propagation of a heritable active state of H19, p57Kip2, Peg3, and Zac1 induced by inhibitors of DNA methylation. AG and PG cells initially treated for 3 days with AzaC, AzadC, or TSA (lanes P1) were cultured for 7 days (lanes P2) or 15 days (lanes P4) after withdrawal of the inhibitors. RNA samples from passages P1, P2, and P4 were analyzed by RT-PCR. The control RNA from untreated uniparental (U) and wild-type (WT) MEFs is indicated. The induction of H19, p57Kip2, and Mash2 was examined in AG MEFs and that of Igf2, Peg3, Zac1, Peg1, Sgce, and Hprt in PG MEFs. The RNA from wild-type (WT) MEFs was used as a positive control for all imprinted genes except in Mash2 the RNA was from placenta.
with inhibitors of histone deacetylases or DNA methyltransferases resulted in activation of only a subset of imprinted genes. In PG cells, both Zac1 and Peg3 were reactivated in cells treated with AzaC or AzadC, whereas Igf2 was induced only by TSA. In AG MEFs, p57Kip2 was synergistically activated by combination of both AzaC and TSA, and H19 was induced after AzadC treatment but not with TSA. However, under identical experimental conditions Grb10, Peg1, Sgce, Snrpn, and U2af1, as well as the tissue-specific imprinted genes Rasgrf1 and Mash2, remained silent after all treatments, demonstrating that not all imprinted genes are responsive to changes in DNA methylation or histone acetylation levels in somatic tissues.

Recently DNA methylation and histone deacetylation have been functionally linked to transcriptional repression. Methyl-CpG-binding proteins MeCP2 (37, 38), MBD2 (39), and MBD3 (40, 41) reside in multiprotein complexes with histone deacetylases. These complexes assemble on methylated DNA mediating transcriptional repression through chromatin hypoacetylation with the silent state being partially reversed by mediating transcriptional repression through chromatin hyd-CpG-binding proteins MeCP2 (37, 38), MBD2 (39), and been functionally linked to transcriptional repression. Meth-in DNA methylation or histone acetylation levels in somatic system. Pedone and Boniello (45) suggested that loss in DNA methylation in PG MEFs (Fig. 5) did not result in detectable changes in expression of these imprinted genes. Also, the observed differential methylation between the expressed and silent alleles might be a consequence and not the cause of silencing. In this case, DNA demethyllysis would not affect the silencing. Detailed analysis of chromatin structure and composition may help elucidate the mechanisms orchestrating allele-specific silencing of this group of imprinted genes.

Propagation of epigenetic states of expression during multiple cell divisions is essential for the stability of imprinting. Remarkably, we observed that the activated state of H19, p57Kip2, Peg3, and Zac1 mediated by DNA demethylation was enriched and stably propagated through multiple mitoses (Fig. 6). In contrast, the activated state of Igf2 and p57Kip2, induced by transient inhibition of histone deacetylase, was quickly reversed after withdrawal of TSA from the culture medium, suggesting that the histone deacetylase activity associated with silencing Igf2 and p57Kip2 is only temporarily inhibited but not disrupted by TSA treatment. These results support a previous observation (4) that changes in DNA methylation, but not histone acetylation, create a heritable epigenetic state at some, but not all, imprinted loci in somatic cells.

Here, we have shown that uniparental AG and PG MEFs provide a unique model to study the regulation of imprinted gene expression in vitro. In these lines, as in the whole mouse, imprinted genes are expressed according to their parental origin. Among the imprinted genes examined, four were responsive to partial loss of methylation (i.e., H19, p57Kip2, Peg3, and Zac1), suggesting that DNA methylation is the primary silencing mechanism for this set of imprinted genes and that additional mechanisms may regulate silencing of other imprinted genes in somatic cells.

Acknowledgments—We thank Lidia Hernandez and Lori Sewell for excellent technical assistance; Camillynn I. Brannan and Shoichi Sunahara for Snrpn and U2af1 probes; Matthieu Gerard for stimulating discussions; and Amar Klar, Jacob Z Dalgaard, and Peter Johnson for suggestions and comments on the manuscript.

REFERENCES

1. Barton, S. C., Surani, M. A., and Norris, M. L. (1984) Nature 311, 374–376
2. Mcgrath, J., and Solter, D. (1984) Cell 37, 179–183
3. Surani, M. A., Barton, S. C., and Norris, M. L. (1986) Cell 45, 127–136
4. Tucker, K. L., Beard, C., Daussmann, J., Jackson-Grusby, L., Laird, P. W., Lei, H., Li, E., and Jaenisch, R. (1996) Genes Dev. 16, 1098–1102
5. Surani, M. A. (1998) Cell 93, 309–312
6. Jaenisch, R. (1997) Trends Genet. 13, 323–329
7. Constancia, M., Pickard, B., Kelsey, G., and Reik, W. (1998) Genome Res. 8, 881–900
Reactivation of Imprinted Genes in Uniparental MEFs

8. Tremblay, K. D., Duran, K. L., and Bartolomei, M. S. (1997) Mol. Cell. Biol. 17, 4322–4329
9. Brannan, C. I., and Bartolomei, M. S. (1999) Curr. Opin. Genet. Dev. 9, 164–170
10. Li, E., Beard, C., and Jaenisch, R. (1993) Nature 366, 362–365
11. Brandeis, M., Kafri, T., Ariel, M., Chaillet, J. R., McCarrery, J., Razin, A., and Cedar, H. (1993) EMBO J. 12, 3669–3677
12. Olek, A., and Walter, J. (1997) Nat. Genet. 17, 275–276
13. Razin, A., and Shemer, R. (1995) Hum. Mol. Genet. 4, 1751–1755
14. Caspary, T., Cleary, M. A., Baker, C. C., Guan, X. J., and Tilghman, S. M. (1996) Mol. Cell. Biol. 16, 3466–3474
15. Stewart, C. L., Vanek, M., and Wagner, E. F. (1985) EMBO J. 4, 3701–3709
16. Mann, J. R., Gadi, I., Harbison, M. L., Abbondanzo, S. J., and Stewart, C. L. (1996) EMBO J. 15, 6251–6260
17. Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994) Manipulating the Mouse Embryo. A Laboratory Manual, 2nd Ed, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Gilliland, G., Perrin, S., Blanchard, K., and Bunn, H. F. (1996) Proc. Natl. Acad. Sci. U. S. A. 87, 2725–2729
19. El Kharroubi, A., and Martin, M. A. (1996) Mol. Cell. Biol. 16, 2958–2966
20. DeChiara, T. M., Robertson, E. J., and Efstratiadis, A. (1991) Cell 64, 849–859
21. Szabo, P. E., and Mann, J. R. (1995) Genes Dev. 9, 1857–1868
22. Kaneko-Ishino, T., Kuroiwa, Y., Miyoshi, N., Kohda, T., Suzuki, R., Yokoyama, M., Viville, S., Barton, S. C., Ishino, F., and Surani, M. A. (1995) Nat. Genet. 11, 52–59
23. Jeff, S. E., Brannan, C. I., Reed, M. L., Ozolik, T., Francke, U., Copeland, N. G., and Jenkins, N. A. (1992) Nat. Genet. 2, 259–264
24. Hayashizaki, Y., Shihata, H., Hirotsune, S., Sugino, H., Okazaki, Y., Sasaki, N., Hirose, K., Imoto, H., Okuzumi, H., Muramatsu, M., Kmatsumura, H., Shirai, T., Moriwaki, K., Katsuki, M., Hatano, H., Sasaki, H., Ueda, T., Mise, N., Takagi, N., Flass, C., and Chapman, V. M. (1994) Nat. Genet. 6, 33–40
25. Piras, G., El Kharroubi, A., Kozlov, S., Escalante-Alcaide, D., Hernandez, L., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Stewart, C. L. (2000) Mol. Cell. Biol. 20, 3308–3315
26. Bartolomei, M. S., Zemel, S., and Tilghman, S. M. (1991) Nature 351, 153–155
27. Miyoshi, N., Kuroiwa, Y., Kohda, T., Sibata, H., Yonekawa, H., Kawabe, T., Hasegawa, H., Barton, S. C., Surani, M. A., Kaneko-Ishino, T., and Ishino, F. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1102–1107
28. Hataf, I., and Mukai, T. (1995) Nat. Genet. 11, 204–206
29. Barlow, D. P., Stoger, R., Herrmann, B. G., Saito, K., and Schweifer, N. (1991) Nature 349, 84–87
30. Plask, C., Shihata, H., Kato, S., Sasaki, H., Hirotsune, S., Okazaki, Y., Held, W. A., Hayashizaki, Y., and Chapman, V. M. (1996) Nat. Genet. 14, 106–109
31. Guilmot, F., Caspary, T., Tilghman, S. M., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Anderson, D. J., Joyner, A. L., Rossant, J., and Nagy, A. (1995) Nat. Genet. 9, 235–242
32. Yoshida, M., Kijima, M., Akita, M., and Beppu, T. (1999) J. Biol. Chem. 265, 17174–17179
33. Kozarides, T. (1999) Curr. Opin. Genet. Dev. 9, 40–48
34. Mizzen, C. A., and Alliss, C. D. (1998) Cell Mol. Life Sci. 54, 6–20
35. Turner, B. M. (2000) Bioessays 22, 836–845
36. Bestor, T. H., and Verdine, G. L. (1994) Curr. Opin. Cell Biol. 6, 380–389
37. Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. (1998) Nature 393, 386–389
38. Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J., and Wolffe, A. P. (1998) Nat. Genet. 19, 187–191
39. Ng, H. H., Zhang, Y., Hendrich, B., Johnson, C. A., Turner, B. M., Eerdjumut-Bromage, H., Tempst, P., Reinberg, D., and Bird, A. (1999) Nat. Genet. 23, 58–61
40. Zhang, Y., Ng, H. H., Eerdjumut-Bromage, H., Tempst, P., Bird, A., and Reinberg, D. (1999) Genes Dev. 13, 1924–1935
41. Wade, P. A., Geggone, A., Jones, P. L., Ballestar, E., Aubry, F., and Wolffe, A. P. (1999) Nat. Genet. 23, 62–66
42. Shemer, R., Birger, Y., Riggs, A. D., and Razin, A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10267–10272
43. Shin, J. Y., Kim, H. S., Park, J., Park, J. B., and Lee, J. Y. (2000) Cancer Res. 60, 262–265
44. Eversole-Cire, P., Ferguson-Smith, A. C., Sasaki, H., Brown, K. D., Cattanach, B. M., Gonzalez, F. A., Surani, M. A., and Jones, P. A. (1993) Mol. Cell. Biol. 13, 4928–4938
45. Pedone, P. V., Pikaart, M. J., Cerrato, F., Vernucci, M., Ungaro, P., Murer, B. R., and Croce, C. M. (1991) FEBS Lett. 282, 45–50
46. Hu, J. F., Nguyen, P. H., Pham, N. V., Vu, T. H., and Hoffman, A. B. (1997) Mol. Endocrinol. 11, 1891–1898
47. Hatafad, I., Iizigawa, K., Yamaoka, T., Wang, X., Arai, Y., Hashido, K., Ohishi, S., Masuda, J., Ogata, J., and Mukai, T. (1995) Nucleic Acid Res. 23, 36–41
48. Lester, S. C., Korn, N. J., and DeMars, R. (1982) Somatic Cell Genet. 8, 265–284