

Embryonic but Not Postnatal Reexpression of Hepatocyte Nuclear Factor 1 α (HNF1 α) Can Reactivate the Silent Phenylalanine Hydroxylase Gene in HNF1 α -Deficient Hepatocytes

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The failure to transcribe the phenylalanine hydroxylase (PAH) gene in the liver of hepatocyte nuclear factor 1 α (HNF1 α)-deficient mice correlated with DNA hypermethylation and the presence of an inactive chromatin structure (M. Pontoglio, D. M. Faust, A. Doyen, M. Yaniv, and M. C. Weiss, *Mol. Cell. Biol.* 17:4948–4956, 1997). To evaluate the precise role played by HNF1 α , DNA methylation, or histone acetylation in PAH gene silencing, we examined conditions that could restore PAH gene expression in HNF1 α -deficient hepatocytes. We show that reactivation of PAH transcription can be achieved by reexpression of HNF1 α in embryonic (i.e., embryonic day 12.5 [e12.5] to e13.5) hepatocytes but not in fetal (e17.5), newborn, and adult HNF1 α -deficient hepatocytes. This defines a temporal competence window during which HNF1 α can act to (re)program PAH gene transcription. We also show that PAH gene silencing can be partially relieved in HNF1 α -deficient hepatocytes by treatment with the demethylating agent 5-azacytidine, even in the absence of HNF1 α . Treatment using 5-azacytidine combined with trichostatin, a histone deacetylase inhibitor, resulted in a synergistic reactivation of the silenced PAH gene in adult hepatocytes, but this activity was not further increased by HNF1 α reexpression. These results suggest that the HNF1 α homeoprotein is involved in stage-specific developmental control of the methylation state and chromatin remodeling of the PAH gene.

During the process of development, genes undergo selective activation, repression, and/or silencing. Some genes are totally silenced, whereas others remain active or potentially active. Failure to activate or repress genes appropriately during development can compromise survival. Thus, correct regulation of gene expression, i.e., tissue-specific expression at appropriate times or in response to specific signals, is essential both to normal development and to correct functioning of the adult organism. Tissue-specific and developmental expression patterns are accompanied by distinct alterations in chromatin structure and DNA methylation status (37). DNA is packaged into either transcriptionally competent euchromatin or repressive, transcriptionally silent heterochromatin. This permits only a small portion of the genome to be expressed in any given cell or tissue type. A strong correlation among DNA methylation, transcriptional silencing, and tightly compacted chromatin structures has been established in many different systems (reviewed in reference 37). The transcriptional repression associated with DNA methylation has been linked to alterations in local chromatin structure leading to the formation of condensed chromatin regions. DNA methyltransferases and methyl-CpG-binding proteins influence local histone acetylation by recruiting histone deacetylase complexes which close chroma-

tin structure, rendering regulatory regions inaccessible to the transcriptional machinery (12, 19, 31, 40). This process prevents regulatory factors from accessing methylated sequences and allows the stable maintenance of gene silencing. Thus, DNA methylation may serve as a unique mechanism for setting up local histone deacetylation, to maintain an epigenetic repressed chromosomal state.

The study of liver-specific gene expression has identified several tissue-enriched transcription factors which act in concert with ubiquitous transcription factors to regulate liver-specific promoters (5). One of these factors is the homeoprotein hepatocyte nuclear factor 1 α (HNF1 α), whose expression is restricted to the liver, pancreas, kidneys, and digestive tract. Disruption of the murine HNF1 α gene results in a complex pattern of traits caused by liver, renal, and pancreatic dysfunctions (33, 36). Interestingly, HNF1 α -deficient mice display hyperphenylalaninemia caused by the lack of hepatic expression of the phenylalanine hydroxylase (PAH) gene. In contrast to PAH, the transcriptional activity of many other known HNF1 α target genes was only partially affected. Two DNase I-hypersensitive sites (HSSII and HSSIII) containing binding sites for HNF1 α were mapped in the PAH transcriptional control region. However, mutation of these sites had little effect on the basal transcriptional activity of the PAH promoter-enhancer sequences linked to a reporter gene in transient-transfection assays (11). The PAH transcriptional defect in the livers of HNF1 α -deficient animals was correlated with the absence of an open chromatin configuration and was accompanied by hypermethylation of the PAH promoter-enhancer regions (34). Furthermore, the failure to activate this gene in

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HNF1 α -deficient mice with its-inducers glucocorticoids and cyclic AMP suggested that an initial transcriptionally competent active state created by HNF1 α binding is a prerequisite step to allow PAH gene modulation in the liver.

In the present study, we investigated the mechanisms by which a single transcription factor is implicated in the formation of an open chromatin configuration and the maintenance of unmethylated regulatory sequences. We examined if and to what extent HNF1 α reexpression, DNA demethylation, or inhibition of histone deacetylation could restore PAH expression in HNF1 α -deficient hepatocytes. We show that HNF1 α reexpression in embryonic (i.e., embryonic day 12.5 [e12.5] to e13.5) HNF1 α -deficient hepatocytes could partially restore PAH gene transcription, whereas fetal (e17.5) newborn, and adult HNF1 α -deficient hepatocytes were refractory to HNF1 α action. Demethylation of the PAH gene locus by 5-azacytidine (5-AzaC) treatment of newborn mice could also restore a low level of transcription. This could be further stimulated by inhibition of histone deacetylation but not by HNF1 α reexpression. These data suggest that HNF1 α plays an important role in the onset of the PAH gene activation via the maintenance of unmethylated status of promoter-enhancer regions during liver development.

MATERIALS AND METHODS

Mouse hepatocyte isolation, cell culture, and transfection. Primary hepatocytes were isolated from adult wild-type and HNF1 α -deficient mice by a two-step in situ collagenase perfusion procedure adapted to mouse livers (2). Hepatocytes from e12.5, e13.5, and e17.5 embryos and newborns were obtained by collagenase digestion of sliced liver, as described previously (4). The enriched hepatocyte suspensions were then purified through an isodensity percoll centrifugation procedure (24). Cell viability was assessed by the trypan blue exclusion test and was always higher than 90%. Hepatocytes were seeded at a density of 10⁵ cells/cm² on rat tail collagen type I-coated culture dishes and were cultivated in Williams' E medium (Gibco/BRL) supplemented with penicillin (100 U/ml), streptomycin (100 μ g/ml), 20 mM HEPES, 2 g of bovine serum albumin/liter, 10 μ M dexamethasone, 1 μ M hydrocortisone, 5 μ g of insulin/ml 5 μ g of transferrin/ml, 5 ng of selenious acid/ml, 0.2 μ g of glucagon/ml, 10 ng of epidermal growth factor/ml, and 2% (vol/vol) dimethyl sulfoxide (DMSO). After 2 h of attachment, the medium was changed and adult hepatocytes were transfected by the lipofection method using the DOTAP transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. Four micrograms of the reporter plasmid β 28₃ containing multimerized HNF1 binding sites was used for each transfection. The medium containing the liposome-DNA complex was replaced 16 h after transfection with fresh medium, and hepatocytes were harvested 24 h later for chloramphenicol acetyltransferase (CAT) assay as described previously (11).

Isolation of total RNA and Northern blot analysis. Total RNA from liver or cultured primary hepatocytes was isolated by the guanidinium thiocyanate-acid phenol method as previously described (6). For Northern blots, 15 μ g of RNA was separated by formaldehyde-agarose gel electrophoresis and transferred to a nylon membrane (Hybond N; Amersham) as recommended by the manufacturer. Probes were labeled by random priming and corresponded to a fragment of the murine PAH cDNA (kindly provided by Daniela Faust) and to a fragment corresponding to the β -actin cDNA.

Infection with recombinant adenovirus. Recombinant adenovirus containing the cDNA for human HNF1 α was obtained by the method described previously (9). Briefly, the human HNF1 α cDNA under the control of the cytomegalovirus (CMV) immediate-early promoter was integrated into the adenovirus backbone by homologous recombination in *Escherichia coli*. Then, the recombinant adenoviral construct was cleaved with *PacI* and transfected into the packaging cell line 293. The recombinant adenovirus expressing the green fluorescent protein (GFP) under the control of the CMV promoter (Quantum Biotechnologies) was used as the control. The adenoviruses were amplified in the 293 cells and purified by cesium chloride density centrifugation; viral titers were estimated optically by the absorbance at 260 nm. HNF1 α -deficient hepatocytes were infected by incubation with the virus in a minimal volume for 1 h at 37°C, with increasing

multiplicity of infection varying by 1 log before addition of extra medium. Nuclear extracts from infected cells were prepared 36 h later and analyzed by Western blotting (7) to define the multiplicity that restores the HNF1 α level found in cultured primary mouse hepatocytes.

In vivo 5-AzaC treatment of mice. Mice weighing about 10 g were injected intraperitoneally with 5-AzaC twice (25 μ g/injection) at days 11 and 14 and were sacrificed at day 25 after birth (16). Control mice were injected with the same volume of saline.

Acid extraction of proteins from TSA-treated hepatocytes. Acid-soluble proteins were prepared from nuclei of primary cultured hepatocytes treated for 48 h with increasing amounts of trichostatin (TSA) (0, 100, and 300 ng/ml). The nuclear pellets were resuspended in 10 volumes of protein extraction buffer (50 mM Tris-HCl [pH 8], 50 mM NaCl, 4 mM MgCl₂, 10 mM sodium butyrate, 5 mM 2-mercaptoethanol, 0.34 M sucrose, 0.5% [vol/vol] Nonidet P-40, and Complete protease inhibitor cocktail [Roche Molecular Biochemicals]). Sulfuric acid was added dropwise to a final concentration of 0.25 M with gentle vortexing, and the nuclear lysate was incubated on ice for 1 h, with vigorous vortexing every 10 min. The lysates were centrifuged at maximum speed, and the supernatant fraction was used for analysis of histone acetylation level by Western blot analysis using anti-acetyl-lysine antibodies (Upstate Biotechnology).

RT-PCR analysis. To eliminate genomic DNA contamination, total RNA (5 μ g) was treated with RNase-free DNase I (Roche Molecular Biochemicals) and then was reverse transcribed into cDNAs using Superscript reverse transcriptase (Gibco/BRL) in the presence of random hexamers. PCR amplifications were performed in the presence of [α -³²P]dATP with the following forward and reverse specific primers: for PAH, ACAGAGGAGGAGAGGAAGAC and TCATAGCGAACGGAGAAG; for HNF4 α , CTCCTTCTTCATGCCAG and ACACGTCCCCATCTGAAG; for HNF3 β , GCCTGAGCCGCGCTCGGGAC and GGTGCAGGTCCAGAAGGAG; for human HNF1 α (hHNF1 α), GCCTGGCCTCCACGAGGCAC; and CTGCTTGGTGGGCGTAGGCT; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CACCATCTCCAGGAGCGAG, and ACAGCCTTGGCACCAGT. For each RNA preparation, we carried out separate amplification products using reverse transcription (RT)-positive and RT-negative reaction mixtures as templates. For PCR amplification, all samples were heated to 94°C for 5 min and then amplified for an optimized number of cycles consisting of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min. All reaction mixtures were then incubated at 72°C for 10 min and cooled to 4°C. PCR products were resolved by 5% polyacrylamide gel electrophoresis and visualized by autoradiography. Signals were collected using a PhosphorImager and quantitated using ImageQuant (Molecular Dynamics).

RESULTS

The mouse PAH gene is expressed in primary hepatocyte culture. To explore the basis for PAH gene regulation and determine the conditions required to reactivate the silent PAH gene in the livers of HNF1 α -deficient mice, we developed an in vitro system based on primary hepatocyte culture. It is known that freshly isolated hepatocytes in culture exhibit a rapid decrease in the transcription of liver-specific genes (8). In order to circumvent and reduce this constraint, we made use of a culture system involving rat tail collagen-coated plates and chemically defined medium supplemented with 2% DMSO (17). It has been previously shown that the expression of many liver-specific genes is maintained for several weeks at 21 to 72% of their hepatic levels when rat hepatocytes are cultured under such conditions (18). To assess the expression level of the PAH gene in primary murine hepatocyte cultures, we performed Northern blot analysis using total RNA extracted at different times after the establishment in culture. As shown in Fig. 1, the level of PAH mRNA decreased immediately after plating. However, the expression level increased at later times, approaching 40% of the level observed at the time of plating (Fig. 1).

Reexpression of HNF1 α in adult HNF1 α -deficient hepatocytes fails to activate PAH expression. It has been previously shown that disruption of the HNF1 α gene resulted in hepatic

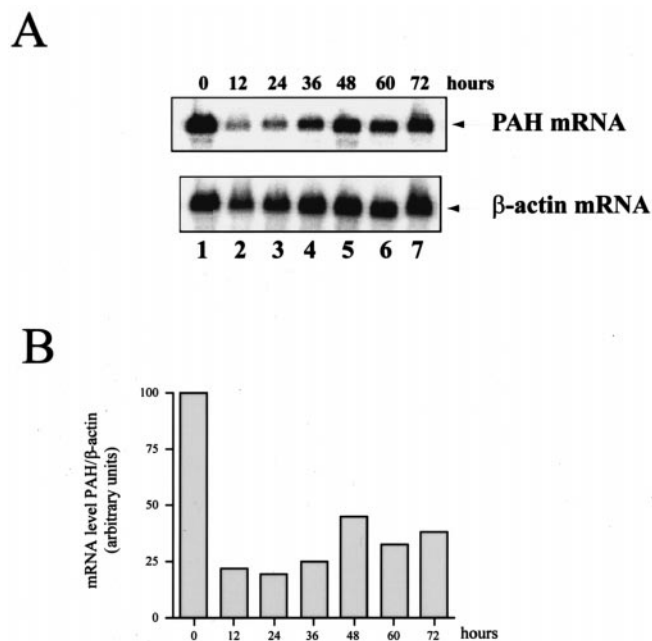


FIG. 1. PAH mRNA levels in primary cultures of mouse hepatocytes. (A) Primary hepatocytes isolated from adult mice were cultured in supplemented serum-free medium containing 2% DMSO on collagen plates for various lengths of time (0, 12, 24, 36, 48, 60, and 72 h). Total RNA was extracted, and the level of the PAH mRNA was monitored by Northern blot analysis. β -Actin mRNA hybridization of the same blot was performed to verify loading of the samples. (B) Shown are PAH mRNA levels normalized for the corresponding β -actin signal.

silencing of the PAH gene (33). Silencing was associated with a modified chromatin structure and DNA methylation pattern (34). The presence of several HNF1 binding sites in DNase I-hypersensitive regions of the PAH promoter-enhancer sequences (11) prompted us to investigate the possibility of reactivating this gene by HNF1 α reexpression. To this end, we reexpressed HNF1 α in primary cultures of adult HNF1 α -deficient hepatocytes using a recombinant adenovirus expressing the hHNF1 α protein (AdhHNF1 α) under the control of the CMV promoter. As a control, we used a recombinant adenovirus expressing GFP (AdGFP). Infection of primary cultures of HNF1 α -deficient hepatocytes with the AdhHNF1 α virus resulted in the synthesis of HNF1 α at levels similar to those found in primary hepatocytes (Fig. 2A). This protein was functionally active, since it activated transcription of a transfected reporter gene containing three multimerized HNF1 binding sites (Fig. 2B). However, to our surprise, HNF1 α failed to activate the silent endogenous PAH gene in adult HNF1 α -deficient hepatocytes (Fig. 3). This result suggested that the closed chromatin configuration of the PAH gene present in HNF1 α -deficient hepatocytes prevented HNF1 α from reactivating the endogenous PAH gene.

Reexpression of HNF1 α in embryonic HNF1 α -deficient hepatocytes is sufficient to activate PAH expression. We figured that the nonpermissive configuration might have been established during embryonic development in the absence of HNF1 α . Low levels of PAH mRNA are detectable from day 11.5 of mouse development, and a major burst in mRNA

synthesis occurs just after birth (reference 34 and data not shown). To verify whether earlier reintroduction of HNF1 α could restore gene activity, we infected hepatocytes isolated from newborn and e17.5 HNF1 α -deficient mice. Again, we failed to reactivate the PAH gene after infection by AdhHNF1 α (data not shown). To test if the PAH gene can be reactivated during earlier liver developmental stages, we in-

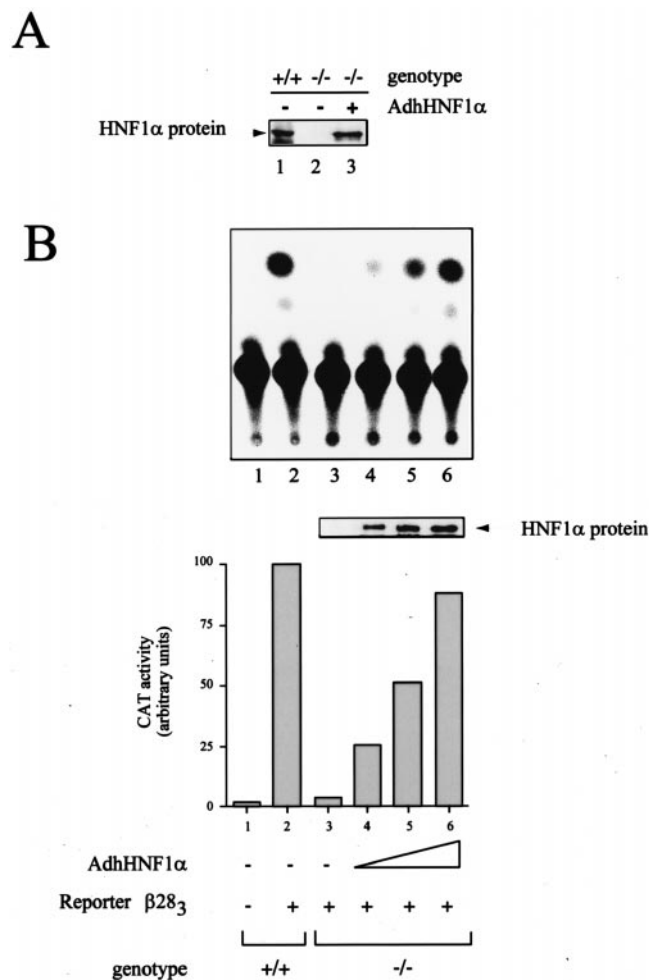


FIG. 2. Adenoviral expression of HNF1 α in HNF1 α -deficient hepatocytes. (A) Primary hepatocytes isolated from adult HNF1 α -deficient mice were infected with HNF1 α - or GFP-expressing adenoviruses at a multiplicity of infection of 10 PFU/cell for 1 h at 37°C. After 36 h of culture, HNF1 α expression levels were determined by Western blot analysis. Nuclear extracts prepared from wild-type hepatocytes (lane 1) or from HNF1 α -deficient hepatocytes infected with either AdGFP (lane 2) or AdhHNF1 α (lane 3) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nylon membrane, and blotted with rabbit polyclonal anti-HNF1 α antibody. (B) Wild-type and HNF1 α -deficient primary hepatocytes were transfected with the reporter construct β 28 $_3$ CAT, containing three HNF1 binding sites upstream of the β -fibrinogen minimal promoter. Twelve hours later, HNF1 α -deficient hepatocytes were infected with increasing amounts of AdhHNF1 α (relative multiplicities of infection of 1, 5, and 10 PFU/cell). Thirty hours later, extracts were prepared and analyzed for HNF1 α protein levels and for CAT activity. HNF1 α expression levels in infected HNF1 α -deficient hepatocytes are presented in the middle panel. A typical CAT assay is shown in the upper panel, and quantification is presented in the lower panel.

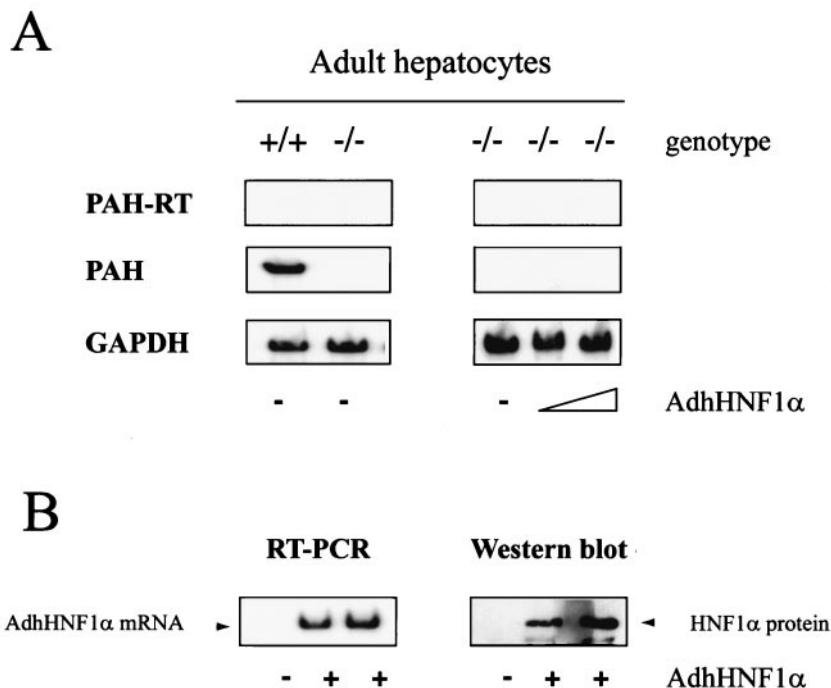


FIG. 3. PAH mRNA expression in adult HNF1 α -deficient hepatocytes after infection with AdhHNF1 α . (A) Primary hepatocytes isolated from adult HNF1 α -deficient mice were infected with increased amounts of AdhHNF1 α (relative multiplicities of infection of 1 and 10 PFU/cell). After 48 h of culture to allow full expression of the adenovirus-transferred genes, total RNA was extracted and levels of PAH and GAPDH mRNA were assayed by semiquantitative RT-PCR. (B) Efficiency of adenoviral infection was monitored in infected HNF1 α -deficient hepatocytes by measuring AdhHNF1 α mRNA expression by RT-PCR analysis using primers specific for the hHNF1 α cDNA and by revealing the presence of the HNF1 α protein by Western blot analysis.

ected hepatocytes isolated from e12.5 HNF1 α -deficient embryos. Remarkably, at this stage of liver development, reintroduction of HNF1 α was sufficient to induce PAH gene transcription (Fig. 4A). The level of PAH reactivation is almost 30% of the PAH expression level of wild-type hepatocytes. To define the critical window for cellular competence, we infected hepatocytes isolated from e13.5 HNF1 α -deficient embryos. At this stage of development, embryonic hepatocytes were still competent for PAH gene reactivation upon addition of exogenous HNF1 α (Fig. 4B). These results suggest that the presence of HNF1 α during early organogenesis is essential for PAH gene activation and its maintenance in an active chromatin configuration.

Effect of histone-hyperacetylating drugs. It is possible that the silent or closed chromatin structure of the PAH gene in fetal, newborn, or adult HNF1 α -deficient hepatocytes prevented the incoming HNF1 α from binding to its target sites. It is generally believed that inactive chromatin contains nonacetylated histones and that gene activation involves increased nucleosomal histone acetylation. We therefore attempted to render the nucleosomal DNA more accessible to transcription factors by treating the primary hepatocytes with TSA, an inhibitor of histone deacetylases. Treatment of cells with TSA has been shown to result in a genomewide increase in the level of histone acetylation and to alter the expression of a number of genes. Consequently, to test the hypothesis that the PAH gene can be reactivated via changes in histone acetylation, HNF1 α -deficient hepatocytes were treated with increasing

amounts of TSA (0, 100, and 300 ng/ml) and harvested for isolation of histones and RNA. No PAH mRNA could be detected by RT-PCR, even after incubation with high doses of TSA (Fig. 5A) at which a large increase in the amount of acetylated histones can be detected (Fig. 5B). We further tested if reintroducing HNF1 α concomitantly with TSA treatment could restore PAH transcription. As with TSA alone, we could not detect any reactivation of PAH gene transcription under these conditions (Fig. 5A).

Effect of DNA demethylation. DNA methylation has been shown to play a dominant role in determining the transcriptional status of genes. In order to examine the influence of methylation on PAH silencing, we attempted to induce partial demethylation with the nucleotide analogue 5-AzaC. We were unable to do so with primary hepatocytes, since they failed to survive 5-AzaC treatment. Therefore, we delivered the drug directly to 2-week-old animals by intraperitoneal injections. Two weeks of treatment with 5-AzaC partially restored transcription of the endogenous PAH gene, because a weak signal could be detected by semiquantitative RT-PCR analysis of the livers of 5-AzaC-treated HNF1 α -deficient mice (Fig. 6A). The PAH reexpression level reached close to 5% of the PAH expression level observed in the livers of wild-type mice (Fig. 6B). This partial effect of 5-AzaC injection on PAH activation was correlated with the low DNA demethylation pattern observed at the PAH locus using the methylation-sensitive endonuclease *HpaII* (data not shown). Furthermore, we verified that under our experimental conditions, treatment with 5-AzaC did not

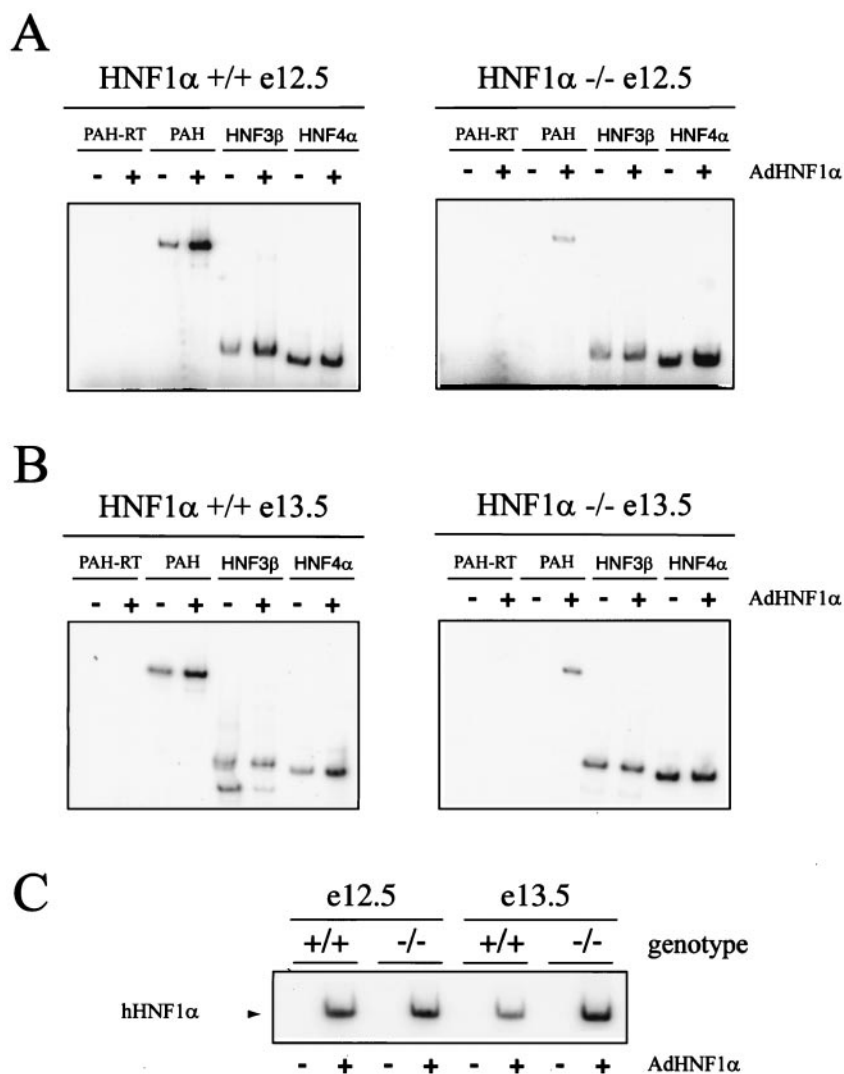


FIG. 4. PAH mRNA expression in e12.5 and e13.5 HNF1 α -deficient hepatocytes after infection with AdhHNF1 α . Primary hepatocytes isolated from e12.5 (A) or e13.5 (B) HNF1 α -deficient hepatocytes were infected with AdhHNF1 α or AdGFP at a multiplicity of infection of 10 PFU/cell. Cells were harvested 48 h later, and PAH, HNF3 β , and HNF4 α mRNA expression levels were determined by semiquantitative RT-PCR analysis. (C) Efficiency of adenoviral infection was monitored by measuring AdhHNF1 α mRNA expression by RT-PCR analysis using primers specific for the human HNF1 α cDNA.

result in the activation of a low level of spurious transcription. We failed to activate the transcription of another HNF1 α -regulated target gene, the kidney-specific Na⁺/glucose cotransporter SGLT2 (35), in wild-type or HNF1 α -deficient hepatocytes (data not shown).

Combined effects of DNA demethylation, histone-hyperacetylating drugs, and AdhHNF1 α infection. It is known that DNA methylation and histone deacetylation play key roles in the silencing of genes. Therefore, we tested whether a combined demethylation and histone deacetylation inhibition could further increase the expression of PAH in HNF1 α -deficient hepatocytes. Indeed, when hepatocytes from 5-AzaC-treated animals were put in culture and treated with TSA, we observed a further threefold increase in the level of PAH mRNA (Fig. 6C, compare bars 1 and 5), demonstrating a certain interaction of both silencing mechanisms. Finally, to

test if expression of HNF1 α could further increase the transcription of the PAH gene after partial reactivation by 5-AzaC treatment, we infected 5-AzaC-treated hepatocytes with increasing amounts of AdhHNF1 α . We could not detect any substantial additional increase in the expression of the endogenous reactivated PAH gene under these conditions (Fig. 6C, bars 2 to 4). Moreover, HNF1 α reexpression in cells treated with both 5-AzaC and TSA did not result in any further increase in PAH mRNA levels (Fig. 6C, bars 6 to 8).

DISCUSSION

It has been previously shown that HNF1 α -deficient mice fail to transcribe the hepatic PAH gene (33), probably because the PAH gene in these mice is hypermethylated and is incorporated into an inactive chromatin conformation (34). These

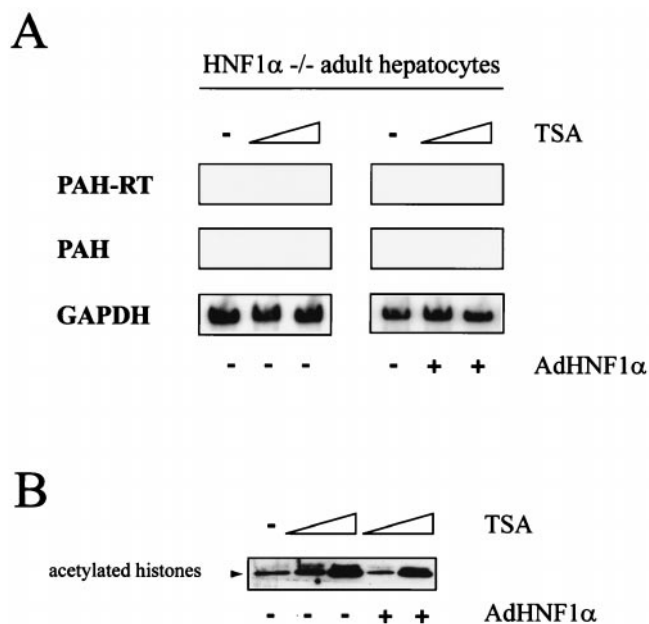


FIG. 5. PAH mRNA expression in adult HNF1 α -deficient hepatocytes after TSA treatment. (A) Primary hepatocytes isolated from adult HNF1 α -deficient mice were treated with increasing amounts of the histone deacetylase inhibitor TSA (0, 100, and 300 ng/ml) and infected with AdhHNF1 α at a multiplicity of infection of 10 PFU/cell. After 48 h of culture, total RNA was extracted, and PAH and GAPDH mRNA levels were monitored by semiquantitative RT-PCR analysis. (B) Examination of histone acetylation levels in TSA-treated hepatocytes was monitored by Western blot analysis. Histones were isolated by acid-soluble nuclear protein preparation and probed with antibodies recognizing acetylated lysines.

observations indicated that HNF1 α must play a crucial role in controlling the expression of this gene. However, the precise role played by HNF1 α in this context has not been elucidated so far. We show here that reexpression of HNF1 α , per se, is sufficient to reactivate transcription from the endogenous PAH locus in embryonic (e12.5 to e13.5) HNF1 α -deficient hepatocytes. In contrast, we failed to restore PAH gene transcription in fetal (e17.5), newborn, and adult HNF1 α -deficient hepatocytes. This failure illustrates the importance of a developmental competence window for reprogramming hepatocyte-specific expression patterns. Our results suggest that the presence of HNF1 α during early liver development is necessary to promote and maintain the formation of a transcriptionally competent PAH locus. This can be achieved by different mechanisms: DNA demethylation and/or chromatin remodeling.

Several liver-enriched transcription factors have been identified whose presence is required for transcription of early hepatic differentiation genes. These factors help impart the competence of hepatic genes to be activated at later stages of development. For example, the transcription factor HNF3 has been implicated in the marking of serum albumin and α -fetoprotein gene enhancers during gut endoderm specification. HNF3 mediates the remodeling of chromatin structure of these genes and thereby promotes the transition between silent and competent chromatin prior to the actual onset of transcription (13). Similarly, transcription factor binding to the β -globin locus control region contributes to developmental and

cell-specific gene expression via localized alterations in chromatin structures (29). In contrast, in the context of the chicken lysozyme locus, it appears that most chromatin pattern formations are set during cellular differentiation prior to the binding of transcriptional activators (23). These studies indicate that a specific chromatin pattern must be established to permit later gene activation.

DNA methylation is closely associated with several biological processes during vertebrate development. The cause-and-effect relationship between DNA methylation status and transcriptional activity has long been debated. It has now been established that gene expression is strongly correlated with the methylation status of DNA (39). In the present study, we show that partial DNA demethylation can restore a low level of PAH transcription, even in the absence of HNF1 α . The observation that 5-AzaC treatment of HNF1 α -deficient mice only poorly reactivates PAH gene transcription can be explained by the mechanism of action of this drug on DNA demethylation. When 5-AzaC is incorporated into DNA upon replication, the modified nucleotide covalently traps DNA methyltransferases (20). In this way, the enzyme is depleted and DNA is then demethylated upon the subsequent rounds of replication. Under normal physiological conditions, most mature hepatocytes are quiescent in the liver. Therefore, the low proliferative index protects most of the hepatocytes from the in vivo effects of 5-AzaC treatment. Stable gene silencing is generally associated with DNA hypermethylation and highly condensed chromatin. It has been suggested that methylated inactive genes contain hypoacetylated histones, whereas unmethylated active genes are preferentially associated with acetylated histones. The link between these two processes was recently established by experiments that showed that DNA methylation can cause transcriptional silencing through local deacetylation of histones (10, 32). Methyl-CpG binding proteins, which bind specifically to methylated DNA, recruit histone deacetylases, which in turn mediate local chromatin remodeling and initiate gene inactivation (19, 30). Hence, one would expect histone deacetylase inhibitors to circumvent the repressive state. However, our results clearly show that TSA, a potent histone deacetylase inhibitor, did not reactivate the PAH gene by itself. This failure may be explained by the level of methylation density (14).

DNA methylation analysis using the *Hpa*II methylation-sensitive endonuclease has revealed that the PAH promoter is heavily methylated in HNF1 α -deficient mice (34). It is known that TSA does not act as a demethylating agent (1, 10), and persisting methylated CpGs could trigger the assembly of repressor complexes insensitive to histone deacetylase inhibitors (38). In agreement with this hypothesis, a recent study on the dynamic nature of DNA methylation and associated transcriptional repression has established that repression observed with low-density methylation is mediated by histone deacetylase activity, whereas the silencing mechanism which acts on densely methylated templates appears to be histone deacetylase independent (26). Moreover, the observation that combined treatment with 5-AzaC and TSA resulted in a further increase in the level of reexpression of the silenced PAH gene suggests that partial demethylation is necessary to establish the TSA-responsive state. This indicates that DNA demethylation could act as a dominant process over histone acetylation in

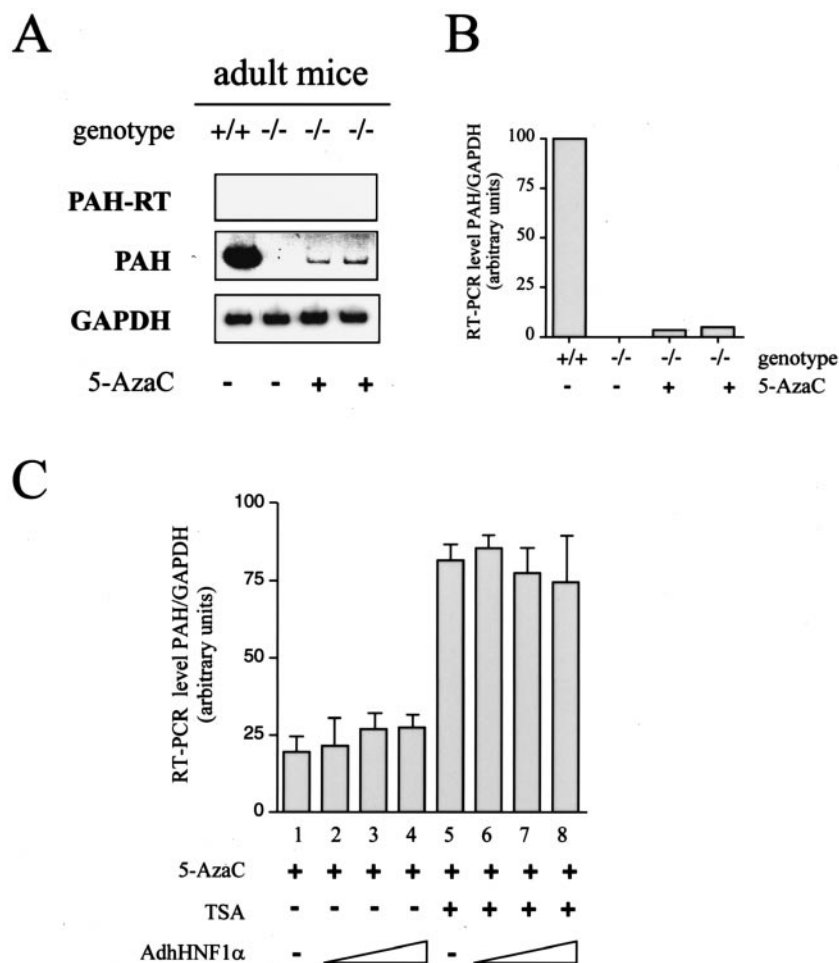


FIG. 6. Injection of 5-AzaC into adult HNF1 α -deficient mice leads to PAH gene reactivation. (A) Two-week-old mice were injected twice at an interval of 3 days intraperitoneally with 5-AzaC (25 μ g/injection). Ten days after the second injection, animals were sacrificed, and hepatic PAH and GAPDH mRNA abundance was monitored by semiquantitative RT-PCR analysis. (B) Quantification of the PAH reexpression level after 5-AzaC treatment, expressed as a ratio of the corresponding GAPDH signal. (C) The combined effect of 5-AzaC injection with AdhHNF1 α infection and TSA treatment on PAH gene reactivation was monitored. Primary hepatocytes isolated from HNF1 α -deficient and 5-AzaC-treated mice incubated in the absence or the presence of 300 ng of TSA per ml were infected with increasing amounts of AdhHNF1 α (relative multiplicities of infection of 1, 5, and 10 PFU/cell). After a 48-h incubation period, total RNA was extracted and semiquantitative RT-PCR analysis was performed to monitor the expression level of PAH and GAPDH mRNAs. The graph shows the quantification of PAH RT-PCR products expressed as a ratio of the corresponding GAPDH signal. The RT-PCR data presented are representative of data obtained with three different animals.

determining silencing at the PAH locus. Our observations are consistent with the results of Cameron et al. (3), who have recently shown that reactivation of methylated endogenous tumor suppressor genes could be achieved only by treatment with 5-AzaC followed by TSA.

It has been postulated that DNA methylation affects transcription either by directly altering the interaction of transcription factors with their binding sites, by altering chromatin structure, or by the combination of both mechanisms (21). The absence of CpG dinucleotides in the consensus binding site for HNF1 α suggests that direct methylation-induced alteration of DNA binding is unlikely to be the primary mechanism by which PAH gene transcription is prevented even upon HNF1 α reexpression. Therefore, DNA methylation may maintain the silent PAH gene by organizing or stabilizing chromatin into a conformation that prevents the accessibility of transcriptional activators to transcription control sequences. In this context,

HNF1 α may be essential at early stages of hepatic differentiation to maintain the unmethylated status of PAH promoter-enhancer regions or promote DNA demethylation. It has been suggested that the establishment and maintenance of "stable" DNA methylation patterns in somatic cells result from a dynamic equilibrium between opposing reactions that promote or inhibit methylation spreading from foci of methylated CpG sites to more distal unmethylated sites. It can be speculated that methylation spreading would occur by decreasing the protective effect conferred by the HNF1 α binding sites, resulting in rapid methylation of the PAH promoter. If the DNA binding by transcription factors plays a role in blocking the spread of methylation once promoter methylation has occurred during differentiation, it should not be easily reversible. The end result of this process would be an epigenetic gene inactivation secondary to DNA methylation and the formation of closed chromatin structures.

A possible molecular link between DNA demethylation processes and transcription factor DNA binding has been postulated recently. It is possible that sequence-specific DNA binding factors might prevent access of the maintenance DNA methyltransferase to these sites, leading to the progressive demethylation of DNA in a locus-specific manner. Such a role has been described for the transcription factor Sp1, which enhances DNA demethylation and prevents de novo methylation of flanking sequences of the adenine phosphoribosyltransferase gene (27). Similarly, the transcription factor NF- κ B has been implicated in a B-cell-specific demethylation process (22). It has been reported that upon transcription factor DNA binding, replication of the target DNA is a prerequisite for efficient demethylation in *Xenopus* embryos (28) and at the replication origin of Epstein-Barr virus (15). Finally, it seems that the affinity of binding of transcription factors to replicating DNA plays a determinant role in the formation of specific DNA demethylation patterns, as was recently demonstrated in the *lac* repressor-operator system (25). Once HNF1 α binds to the PAH promoter-enhancer regions during early liver development, it could contribute to the maintenance of the unmethylated status of the promoter-enhancer PAH gene. Thus, during embryogenesis, the appearance of specific transcription factors and sustained genomic replication could represent a parsimonious mechanism to allow massive promoter-specific demethylation or maintenance of unmethylated tissue-restricted gene status.

Many pathological conditions are associated with altered gene expression patterns. Mutations in transcription factors have profound effects on the onset of diseases. It is critical to determine the molecular mechanisms that allow silent genes to be selectively reactivated, leading toward therapeutic approaches. The potential for gene therapy using transcription factors is enormous and spans many genetic diseases. Advances in prenatal diagnosis and gene transfer technology have allowed consideration of prenatal gene therapy for inborn diseases. A compelling argument can be made for this strategy in treating childhood genetic diseases that are fatal in the prenatal period. In other diseases, the fetal environment may offer unique biological advantages that favor a prenatal gene therapy strategy over treatment after birth. Early therapeutic gene applications may allow targeting of still expanding stem cell populations of organs or cell systems inaccessible later in adulthood. Nevertheless, effective treatment of inborn disorders involving transcription factors is likely to be very complex. As pointed out in this study, the design of rational prenatal therapy cannot neglect the potential problem concerning the competence window of specific transcription factors to reprogram gene expression during development.

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REFERENCES

- Ammerpohl, A., A. Schmitz, L. Steinmuller, and R. Renkawitz. 1998. Repression of the mouse M-lysozyme gene involves both hindrance of enhancer factor binding to the methylated enhancer and histone deacetylation. *Nucleic Acids Res.* **26**:5256–5260.
- Berry, M. N., and D. S. Friend. 1969. High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. *J. Cell Biol.* **43**:506–520.
- Cameron, E. E., K. E. Bachman, S. Myöhänen, J. G. Herman, and S. B. Baylin. 1999. Synergy of demethylation and histone acetylation inhibition in the re-expression of genes silenced in cancer. *Nat. Genet.* **21**:103–107.
- Carpenter, S. P., J. M. Lasker, and J. L. Raucy. 1996. Expression, induction, and catalytic activity of the ethanol-inducible cytochrome P450 (CYP2E1) in human fetal liver and hepatocytes. *Mol. Pharmacol.* **49**:260–268.
- Cereghini, S. 1996. Liver-enriched transcription factors and hepatocyte differentiation. *FASEB J.* **10**:267–282.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159.
- Chouard, T., O. Jeannequin, J. Rey-Campos, M. Yaniv, and F. Traincard. 1997. A set of polyclonal and monoclonal antibodies reveals major differences in post-translational modification of the rat HNF1 and vHNF1 homeoproteins. *Biochimie* **79**:707–715.
- Clayton, D. F., and J. E. Darnell, Jr. 1983. Changes in liver-specific compared to common gene transcription during primary culture of mouse hepatocytes. *Mol. Cell. Biol.* **3**:1552–1561.
- Crouzet, J., L. Naudin, C. Orsini, E. Vigne, L. Ferrero, A. Le Roux, P. Benoit, M. Latta, C. Torrent, D. Branellec, P. Denêfle, J. F. Mayaux, M. Perricaudet, and P. Yeh. 1997. Recombinational construction in *Escherichia coli* of infectious adenoviral genomes. *Proc. Natl. Acad. Sci. USA* **94**:1414–1419.
- Eden, S., T. Hashimshony, I. Keshet, H. Cedar, and A. W. Thorne. 1998. DNA methylation models histone acetylation. *Nature* **394**:842.
- Faust, D. M., A.-M. Catherin, S. Barbaux, L. Belkadi, T. Imazumi-Scherrer, and M. C. Weiss. 1996. The activity of the highly inducible mouse phenylalanine hydroxylase gene promoter is dependent upon a tissue-specific, hormone-inducible enhancer. *Mol. Cell. Biol.* **16**:3125–3137.
- Fuks, F., W. A. Burgers, A. Brehm, L. Hughes-Davies, and T. Kouzarides. 2000. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nat. Genet.* **24**:88–91.
- Gualdi, R., P. Bossard, M. Zheng, Y. Hamada, J. R. Coleman, and K. S. Zaret. 1996. Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. *Genes Dev.* **10**:1670–1682.
- Hsieh, C.-L. 1994. Dependence of transcriptional repression on CpG methylation density. *Mol. Cell. Biol.* **14**:5487–5494.
- Hsieh, C.-L. 1999. Evidence that protein binding specifies sites of DNA demethylation. *Mol. Cell. Biol.* **19**:46–56.
- Hu, J. F., P. H. Nguyen, N. V. Pham, T. H. Vu, and A. R. Hoffman. 1997. Modulation of *Igf2* genomic imprinting in mice induced by 5-azacytidine, an inhibitor of DNA methylation. *Mol. Endocrinol.* **11**:1891–1898.
- Isom, H. C., T. Secott, I. Georgoff, C. Woodworth, and J. Mummaw. 1985. Maintenance of differentiated rat hepatocytes in primary culture. *Proc. Natl. Acad. Sci. USA* **82**:3252–3256.
- Isom, I., I. Georgoff, M. Salditt-Georgieff, and J. E. Darnell. 1987. Persistence of liver-specific messenger RNA in cultured hepatocytes: different regulatory events for different genes. *J. Cell Biol.* **105**:2877–2885.
- Jones, P. L., G. J. C. Venstra, P. A. Wade, D. Vermaak, S. U. Kass, N. Landsberger, J. Strouboulis, and A. P. Wolffe. 1998. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat. Genet.* **19**:187–191.
- Juttermann, R., E. Li, and R. Jaenisch. 1994. Toxicity of 5-aza-2'-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc. Natl. Acad. Sci. USA* **91**:11797–11801.
- Kass, S. U., D. Pruss, and A. P. Wolffe. 1997. How does DNA methylation repress transcription? *Trends Genet.* **13**:444–449.
- Kirillov, A., B. Kistler, R. Mostoslavsky, H. Cedar, T. Wirth, and Y. Bergman. 1996. A role for nuclear NF- κ B in B-cell-specific demethylation of the IgK locus. *Nat. Genet.* **13**:435–441.
- Kontaraki, J., H. Chen, A. Riggs, and C. Bonifer. 2000. Chromatin fine structure profiles for a developmentally regulated gene: reorganization of the lysozyme locus before trans-activator binding and gene expression. *Genes Dev.* **14**:2106–2122.
- Kreamer, B. L., J. L. Staeker, N. Sawada, G. L. Sattler, M. T. S. Hsia, and H. C. Pitof. 1986. Use of low speed, iso-density percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. *In Vitro Cell. Dev. Biol.* **22**:201–211.
- Lin, I. G., T. J. Tomzynski, Q. Ou, and C.-L. Hsieh. 2000. Modulation of DNA binding protein affinity directly affects target site demethylation. *Mol. Cell. Biol.* **20**:2343–2349.
- Lorincz, M. C., D. Schübeler, S. C. Goetze, M. Walters, M. Groudine, and D. I. K. Martin. 2000. Dynamic analysis of proviral induction and de novo methylation: implications for a histone deacetylase-independent, methylation density-dependent mechanism of transcriptional repression. *Mol. Cell. Biol.* **20**:842–850.
- Macleod, D., J. Charlton, J. Mullins, and A. P. Bird. 1994. Sp1 sites in the

- mouse *aprt* gene promoter are required to prevent methylation of the CpG island. *Genes Dev.* **8**:2282–2292.
28. **Matsuo, K., J. Silke, O. Georgiev, P. Marti, N. Giovannini, and D. Rungger.** 1998. An embryonic demethylation mechanism involving binding of transcription factors to replicating DNA. *EMBO J.* **17**:1446–1453.
 29. **McMorrow, T., A. van Den Wijngaard, A. Wollenschlaeger, M. van De Corput, K. Monkhorst, T. Trimborn, P. Fraser, M. van Lohuizen, T. Jenwein, M. Djabali, S. Philipsen, F. Grosveld, and E. Milot.** 2000. Activation of the beta globin locus by transcription factors and chromatin modifiers. *EMBO J.* **19**:4989–4996.
 30. **Nan, X., H.-H. Ng, C. A. Johnson, C. D. Laherty, B. M. Turner, R. N. Eisenman, and A. Bird.** 1998. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393**:386–389.
 31. **Ng, H. H., Y. Zhang, B. Hendrich, C. A. Johnson, B. M. Turner, H. Erdjument-Bromage, P. Tempst, D. Reinberg, and A. Bird.** 1999. MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. *Nat. Genet.* **23**:58–61.
 32. **Pikaart, M. J., F. Recillas-Targa, and G. Felsenfeld.** 1998. Loss of transcriptional activity of a transgene is accompanied by DNA methylation and histone deacetylation and is prevented by insulators. *Genes Dev.* **12**:2852–2862.
 33. **Pontoglio, M., J. Barra, M. Hadchouel, A. Doyen, C. Kress, J. Poggi Bach, C. Babinet, and M. Yaniv.** 1996. Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome. *Cell* **84**:575–585.
 34. **Pontoglio, M., D. M. Faust, A. Doyen, M. Yaniv, and M. C. Weiss.** 1997. Hepatocyte nuclear factor 1 α gene inactivation impairs chromatin remodeling and demethylation of the phenylalanine hydroxylase gene. *Mol. Cell. Biol.* **17**:4948–4956.
 35. **Pontoglio, M., D. Prie, C. Cheret, A. Doyen, C. Leroy, P. Froguel, G. Velho, M. Yaniv, and G. Friedlander.** 2000. HNF1 α controls renal glucose reabsorption in mouse and man. *EMBO Rep.* **1**:359–365.
 36. **Pontoglio, M., S. Sreenan, M. Roe, W. Pugh, D. Ostrega, A. Doyen, A. J. Pick, A. Baldwin, G. Velho, P. Froguel, M. Levisetti, S. Bonner-Weir, G. I. Bell, M. Yaniv, and K. S. Polonsky.** 1998. Defective insulin secretion in hepatocyte nuclear factor 1 alpha-deficient mice. *J. Clin. Investig.* **101**:2215–2222.
 37. **Razin, A.** 1998. CpG methylation, chromatin structure and gene silencing—a three-way connection. *EMBO J.* **17**:4905–4908.
 38. **Schübeler, D., M. C. Lorincz, D. M. Cimborra, A. Telling, Y.-Q. Feng, E. E. Bouhassira, and M. Groudine.** 2000. Genomic targeting of methylated DNA: influence of methylation on transcription, replication, chromatin structure, and histone acetylation. *Mol. Cell. Biol.* **20**:9103–9112.
 39. **Siegfried, Z., S. Eden, M. Mendelsohn, X. Feng, B. Z. Tsuberi, and H. Cedar.** 1999. DNA methylation represses transcription *in vivo*. *Nat. Genet.* **22**:203–206.
 40. **Wade, P. A., A. Geggone, P. L. Jones, E. Ballestar, F. Aubry, and A. P. Wolffe.** 1999. Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. *Nat. Genet.* **23**:62–66.