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Hepatocyte Nuclear Factor 1α Gene Inactivation Impairs Chromatin Remodeling and Demethylation of the Phenylalanine Hydroxylase Gene

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Hepatocyte nuclear factor 1 α (HNF1α) is a homeoprotein that is expressed in the liver, kidney, pancreas, and digestive tract. Its inactivation in mouse resulted in decreased transcription of known target genes such as albumin and α_1-antitrypsin. In contrast, the phenylalanine hydroxylase (PAH) gene was totally silent and unresponsive to normal inducers like glucocorticoids and cyclic AMP in the liver. DNase I and micrococcal nuclease digestion of liver nuclei showed that HNF1α inactivation had drastic effects on the chromatin structure of the PAH regulatory regions. Three DNase I-hypersensitive sites (HSSI, HSSII, and HSSIII), typical of the actively transcribed PAH gene, were undetectable in liver from HNF1α-deficient animals. Both HSSII and HSSIII elements harbor HNF1 sites, but only the latter has detectable enhancer activity in transient-transfection assays. In addition, the PAH promoter in livers of HNF1α-deficient animals was methylated. These results suggest that HNF1α could activate transcription through two mechanisms. One implies participation in the recruitment of the general transcription machinery to the promoter, and the second involves the remodeling of chromatin structure and demethylation that would allow transcription factors to interact with their cognate *cis*-acting elements.

One of the crucial steps in the transcriptional activation process is the rearrangement or disruption of the closed chromatin structure that characterizes the inactive status of a gene. These chromatin transitions result in a different degree of accessibility, which can be empirically monitored by treatment of purified nuclei with DNase I, micrococcal nuclease, or restriction endonucleases. Transcriptionally active genes and their regulatory regions are characterized by a greater general sensitivity and by the presence of sites that are hypersensitive to nucleases (reviewed in references 23 and 28).

Monitoring of the chromatin structure of tissue-specific genes has revealed the existence of DNase I-hypersensitive sites, some of which can be found only in cell types where the corresponding gene is transcribed (20). Very often these tissue-specific DNase I-hypersensitive sites coincide with crucial regulatory elements, i.e., promoters or enhancers, as in the albumin gene (32, 34), or the more complex locus control region described for the human β-globin locus (24, 47). The fact that these hypersensitive sites appear only in nuclei actively transcribing specific genes reflects the presence of nucleoprotein structures that are likely to be involved in the transcriptional activation process. Frequently these structures encompass multiple binding sites for both ubiquitous and cell-specific transcription factors. It is not yet fully established whether some of these factors are involved only in the initial opening of the chromatin structure and others are involved only in the recruitment of the transcriptional machinery or whether these two functions can be accomplished by the same factor. Chromatin opening may require the activity of complexes like snf/swi or nurf (37, 46, 48).

Active genes are also distinguished from inactive genes by the degree of DNA methylation. Hypermethylation of tissue-specific genes is established during implantation. The subsequent tissue-specific gene activation process, underlying cellular differentiation, is accompanied by a programmed gene-specific demethylation (for a review, see reference 19). For the murine phosphoenolpyruvate carboxykinase (PEPCK) gene, a specific and progressive demethylation pattern precedes and accompanies the transcriptional activation occurring around the delivery date (5). Hence, inactive genes are characterized by closed chromatin structures and DNA hypermethylation. Transcriptional activation appears to be a multistep process that involves chromatin remodeling and DNA demethylation. However, the exact mechanisms that govern these two processes are not fully understood.

Several tissue-enriched transcription factors have been identified in the course of the study of liver-specific transcription. These include hepatocyte nuclear factor 1α (HNF1α) and HNF1β (2, 14, 18, 25, 39); HNF3α, HNF3β, and HNF3γ (29, 30); HNF4 (41); and members of the C/EBP (26) and LAP (17) b-Zip families of transcription factors. Together with ubiquitous transcription factors, they have been shown to activate liver-specific promoters and enhancers in vitro or upon transfection. Some of them were shown to interact directly with components of the basic transcriptional apparatus like TFIID, TFIIB, and others (33). These interactions are believed to recruit RNA polymerase II to the initiation sites. In addition, transcription factors may favor the formation of open complexes and promoter escape. HNF1α is a tissue-restricted transcription factor that belongs to the homeoprotein family. In vitro transcription and transfection studies suggested that HNF1α plays an important role in the transcriptional activa-

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tion of a large set of hepatic genes including albumin, α_1-antitrypsin, and α- and β-fibrinogen (reviewed in reference 44). The protein is expressed predominantly in the liver, kidneys, pancreas, and digestive tract (6, 25). All these tissues also express HNF1β, a close homolog of HNF1α (11, 18, 36, 39). However, while HNF1β is as abundant as HNF1α in the kidneys, only traces of HNF1β are present in the liver.

The inactivation of HNF1α by homologous recombination resulted in a complex pattern of traits (38). Mice lacking HNF1α presented with hepatic dysfunction, renal Fanconi's syndrome caused by a renal proximal tubular dysfunction, and phenylketonuria due to the lack of the hepatic expression of phenylalanine hydroxylase (PAH). In contrast to PAH, most of the previously described HNF1 target genes were only partially affected by HNF1α inactivation. For example, the transcription rate of the albumin gene was reduced roughly fourfold. This result paralleled the effect of mutations of the HNF1 binding site located in the albumin promoter described as the proximal element (PE). In fact, mutagenesis of PE, in the context of the promoter that contains more distal elements as well, resulted in a fourfold reduction of reporter gene expression in transient-transfection experiments (43).

The PAH transcriptional control region contains several DNase I-hypersensitive sites. One of them corresponds to a hormone-inducible enhancer containing HNF1 binding sites (22). Since the transcriptional activity of this enhancer is only partially affected by mutations of the HNF1 sites, the complete absence of expression of the gene in HNF1α-deficient mice was enigmatic. In the present work, we have tried to elucidate the mechanisms underlying this phenomenon.

We show that the lack of hepatic PAH expression in HNF1α-deficient animals is correlated with the absence of an "open" chromatin domain in the PAH promoter/enhancer region. Three tissue-specific DNase I-hypersensitive sites, characteristic of the liver, are undetectable in nuclei prepared from livers of HNF1α-deficient animals. These sites also show a potential nucleosomal positioning that is absent in the HNF1α-mutant mice. The lack of hepatic PAH expression is also accompanied by hypermethylation of the promoter.

MATERIALS AND METHODS

Preparation of nuclei. Nuclei were prepared from freshly excised livers, kidneys, and spleens of mice by homogenization in a Teflon pestle tissue homogenizer or in a glass Dounce homogenizer in ice cold 0.3 M sucrose in buffer A (60 mM KCl, 15 mM NaCl, 15 mM HEPES [pH 7.5], 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 14 mM β-mercaptoethanol) in the presence of 0.5% Nonidet P-40 and a cocktail of protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 0.5 μg of aprotinin per ml, 0.5 μg of pepstatin per ml, 0.5 μg of leupeptin per ml). The homogenized suspension was decanted for 5 min on ice, and the supernatant was centrifuged for 5 min at 800 × *g* at 4°C. The nuclear pellet was resuspended in 2 ml of ice-cold 0.3 M sucrose in buffer A and carefully layered on 3 ml of ice-cold 0.9 M sucrose in buffer A. This step gradient was centrifuged for 15 min at 2,000 × *g* at 4°C. The purified nuclei were finally resuspended in RB buffer (buffer A with the addition of 5 mM $MgCl_2$ and without β-mercaptoethanol).

Analysis of DNase I hypersensitivity. Nuclear suspensions (in RB buffer) corresponding to roughly 180 μg of DNA per aliquot were treated for 10 min at 0°C with DNase I (grade I; Boehringer Mannheim) by using between 0 and 11 U per μg of DNA. The nuclei were then lysed and digested with proteinase K. DNA was phenol extracted, precipitated, cleaved with an appropriate restriction enzyme and subjected to Southern blot analysis (15) with probes abutting the restriction cleavage site, and labeled by random priming.

Analysis of micrococcal nuclease sensitivity. Nuclear suspensions corresponding to 300 μg of genomic DNA were digested at room temperature for 0 to 9 min with 0.4 U of micrococcal nuclease in 500 μl of RB buffer in the presence of 5 mM $CaCl_2$. A 50-μl volume of reaction mixture was arrested at different times by the addition of 10 μl of 166 mM EDTA. Following proteinase K digestion, DNA was extracted, digested with the appropriate restriction endonuclease enzyme, and subjected to Southern blot analysis. To control for the micrococcal nuclease sequence-specific cleavage, we carried out, as a control, the digestion of purified liver genomic DNA. To reduce the viscosity of the solution, we predigested the genomic DNA with an appropriate restriction enzyme. Once digested, the DNA was more easily mixed with the micrococcal nuclease enzyme and permitted a more synchronous and uniform cleavage. Hybridizations were performed as described previously (15) with probes labeled by random priming or with single stranded DNA probes obtained by linear PCR amplifications.

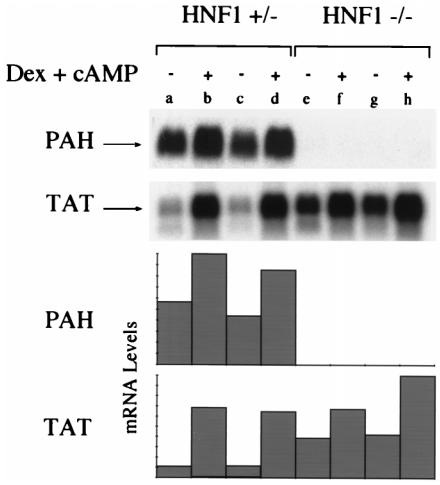

FIG. 1. Dexamethasone (Dex) and cAMP cannot induce PAH expression in HNF1α-deficient animals. Mice injected with dexamethasone and cAMP or with saline were either sacrificed after 3 h (lanes a, b, e and f) or injected again after 12 and 24 h and sacrificed at 27 h (lanes c, d, g, and h). Total hepatic RNA was extracted and analyzed by Northern blotting. The PAH- and TAT-specific bands are indicated by arrows at the left. The histograms represent the PhosphorImager-quantified PAH and TAT signals expressed in arbitrary units.

RNA analysis. Total RNA was isolated as described elsewhere (13). For Northern blots, 15 μg of total RNA was separated in 0.66 M formaldehyde–1% agarose gels and transferred to Hybond N (Amersham) nylon membranes. Probes were labeled by random priming and corresponded to the 640-bp *Pst*I fragment of murine PAH cDNA and to a 2,370-bp fragment corresponding to the entire cDNA coding for TAT.

Hormonal treatment of animals. Mice were injected with dexamethasone (10 μg/100 g of body weight) and with 8-(4-chlorophenylthio)-cyclic AMP (cAMP) (5 mg/100 g of body weight) or with saline solution as controls. Animals were sacrificed 1 and 3 h after the last cAMP and dexamethasone injections respectively. Periodic injections (every 12 h) were carried out when the treatment was prolonged for 27 h.

Nuclear extracts. Nuclear extracts were prepared as described previously (12), except that $(NH_4)_2SO_4$ precipitation and dialysis were omitted. The protein concentrations were determined by the Bradford method (7).

Band shift assays. Double-stranded oligonucleotides were labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. Incubation of the labeled probes (0.4 ng) with 10 μg of nuclear extract from FGC4 cells (1) or with 3 μg of liver or renal nuclear extracts for 10 min in a final volume of 14 μl was followed by electrophoresis in a 6% polyacrylamide gel with 0.25× TBE (22 mM Tris, 22 mM boric acid, 0.5 mM EDTA) and autoradiography. The reaction conditions were as described previously (10). For competition assays, a 50-fold molar excess of unlabeled oligonucleotides was added to the reaction mixtures.

Methylation analysis. DNA extracted from different tissues was digested with *Hin*dIII and with either *Msp*I or *Hpa*II overnight with a 20-fold excess of enzyme. The DNA was subsequently subjected to Southern blot analysis.

RESULTS

Glucocorticoids and cAMP fail to induce hepatic PAH in HNF1α-deficient animals. As mentioned above, the PAH gene is silent in the livers of HNF1α−/− mice. Previous studies have demonstrated that the activity of the PAH transcriptional regulatory region linked to a reporter gene is strongly enhanced by hormones (22). These results led us to examine whether treatment of HNF1α-deficient animals with glucocorticoids and cAMP could activate the expression of PAH in the livers of these animals. Figure 1 shows that dexamethasone and cAMP treatment of heterozygous animals, indistinguishable from their wild-type counterparts in their phenotype, increased the level of PAH mRNA compared to that in the saline-injected

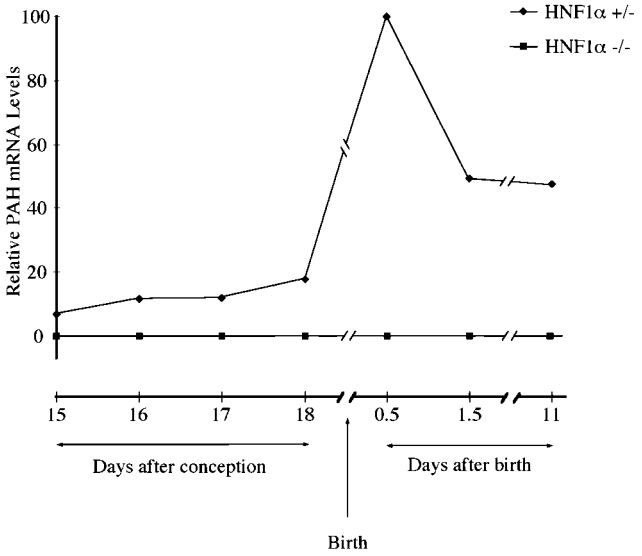

FIG. 2. Hepatic PAH expression during development. The kinetics of PAH mRNA induction was determined by quantification of signals specific for PAH mRNA on Northern blots with total hepatic RNA. The first four points are prenatal, whereas the following three are postnatal, as indicated on the abscissa.

controls. However, the same doses failed to induce any PAH expression in HNF1α-deficient animals.

The lack of activation of PAH transcription upon hormonal induction could be explained by a defect in the hormone responsiveness of HNF1α-deficient animals. To examine this issue, we monitored the expression of tyrosine aminotransferase (TAT), known to be under the control of the same hormones (3). As shown in Fig. 1, TAT is strongly induced in heterozygous animals. TAT basal levels in the HNF1α-deficient mice (saline-injected animals) were significantly higher than in the identically treated control animals. This is likely to be due to the constitutively activated gluconeogenesis that characterizes the HNF1α-deficient animals. These mice experience a massive glucose loss in their urine and must compensate for this severe glucosuria. Nevertheless, the treatment with dexamethasone and cAMP further stimulated the expression of TAT in the mutants, so that transcript levels were the same as for the hormone-treated controls. These results rule out a defect in the general transactivation capacity of the hormone receptors in HNF1α-deficient mice.

The onset of hepatic PAH expression is prenatal. Many hepatic functions, especially those involved in gluconeogenesis, like PEPCK and TAT, are strongly activated during the first days of postnatal life. In fact, the corresponding hepatic mRNA levels increase after birth in response to the hormonal surge that occurs at birth. PAH, like TAT and PEPCK, catalyzes an enzymatic activity that is required for postnatal life.

The possibility that a neonatal PAH mRNA peak exists, analogous to that of PEPCK and TAT, led us to investigate whether PAH could be temporarily induced in newborns in spite of the deficiency of HNF1α. As shown in Fig. 2, the expression of PAH in normal animals was already detectable, at a low level, in embryonic liver by day 15 after conception. On the day of birth, PAH expression increased by a factor of 4 to 5, and then decreased somewhat to remain essentially unchanged until adulthood. Throughout this time, no PAH mRNA could be detected in the livers of HNF1α-deficient mice.

Liver-specific DNase I-hypersensitive sites of the PAH gene are absent in HNF1α−/− animals. In an earlier study, four DNase I-hypersensitive sites were identified in the regulatory region of the mouse PAH gene (22). HSSI corresponds to the transcription start sites, whereas HSSII, HSSIII and HSSIV are located respectively 1.2, 3.5 and 8.8 kb upstream of HSSI. HSSIII colocalizes with a strong glucocorticoid- and cAMP-inducible enhancer (HSIII), which for maximal activity requires HNF1 and C/EBP as well. In transient-transfection assays, tissue-specific transcription activity of the PAH promoter was strictly dependent on the HSIII enhancer. HSSIV is associated with a non-tissue-specific enhancer, while no effects on transcriptional activation could be demonstrated for HSSII-containing sequences (22).

The complete absence of PAH transcription in the liver of HNF1α-deficient animals prompted us to investigate whether the chromatin structure, typical of the PAH promoter in liver, was established in the mutant mice. To this end, we searched for the presence of three of the four previously described DNase I-hypersensitive sites.

Digestion of nuclei from organs of heterozygous control animals (liver, kidney, and spleen) revealed that HSSI, HSSII, and HSSIII were present in the liver and not in the spleen, as expected (Fig. 3). A careful analysis of the kidneys revealed that not only was HSSI present but HSSII and HSSIII were present as well (Fig. 3). However, when nuclei from livers of mutant mice were examined, no trace of any hypersensitive site was detectable. In sharp contrast, kidney chromatin from mutant mice did not show any alteration of the pattern of hypersensitive sites compared to the heterozygous controls (Fig. 3). Finally, to exclude a general deficiency in the development of hypersensitive sites in hepatic HNF1 target genes in mutant animals, we monitored the DNase I-hypersensitive site previously detected in the promoter of the albumin gene (32). The control and mutant animals exhibited the same degree of DNase I hypersensitivity (data not shown).

The HSSII region contains two functional HNF1 binding sites. The observation that HNF1α plays such a crucial role in the transcriptional activation of PAH was unexpected, especially since the two HNF1 binding sites identified in the HSIII enhancer proved not to be essential for basal activity in transfection assays. Consequently, we searched for the existence of additional possible HNF1 binding sites in the PAH regulatory region. Analysis of the sequence 2,200 bp upstream of the initiation sites by a computer-assisted search with a weight matrix for the HNF1 binding site consensus (42) led to the identification of two putative sites located close to one another at around 1.2 kb upstream from the initiation starts. These two HNF1 binding sites colocalize with HSSII, a tissue-specific DNase I-hypersensitive site whose deletion did not influence activity in transient-expression assays (22).

Band shift and competition analysis with nuclear extracts from PAH-expressing hepatoma cells (Fig. 4) confirmed that HNF1 binds to one of these two sites (site 2) with an affinity comparable to that of the strong HNF1 binding site located in the proximal albumin promoter (PE). HNF1 also binds to the second site (site 1) with a low affinity (Fig. 4). The use of a probe containing both sites showed that HNF1α occupancy was not mutually exclusive and that both sites could be simultaneously bound in vitro (data not shown).

A specific micrococcal nuclease cleavage pattern is absent in HNF1α−/− livers. To understand better the mechanism responsible for the absence of PAH transcription and of DNase I hypersensitivity in the regulatory region of the gene, we attempted to map the position of nucleosomes on the PAH promoter by micrococcal nuclease digestion of nuclei and in-

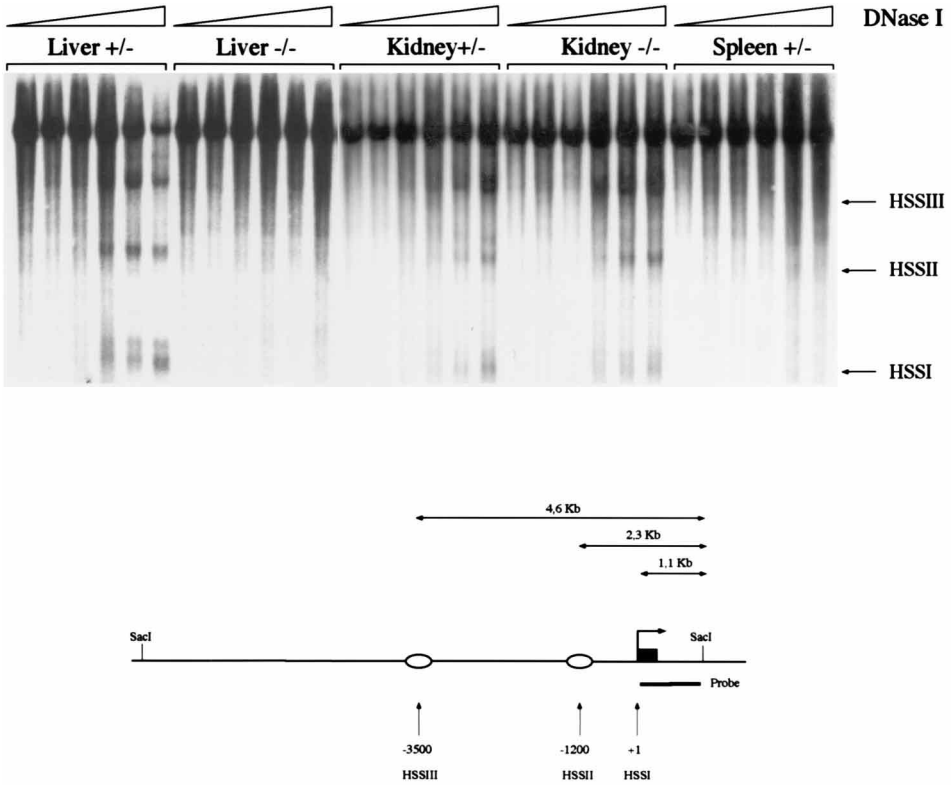

FIG. 3. DNase I hypersensitivity of the PAH promoter-enhancer region. Freshly prepared nuclei from spleen, liver, or kidneys from HNF1+/− or HNF1−/− animals, as indicated on the top, were treated with increasing concentrations of DNase I (0 to 11 U/μg of DNA). Genomic DNA was extracted, digested with *Sac*I, and subjected to Southern blot analysis. Below is indicated the position of DNase I-hypersensitive sites (HSS) and relative distances from the transcription start point. The black box represents the first exon. The bar represents the position of the hybridization probe. It was not feasible to monitor the HSSIV site in the genetic background of the mice, since *Xba*I-digested DNA failed to reveal the expected single band (22) and instead showed multiple bands hybridizing with the probe.

direct end labeling. Different digestion patterns were obtained with wild-type and mutant livers (Fig. 5). Normal liver was characterized by the presence of a hypersensitive region (MNI) located between 1,100 and 1,300 bp upstream of the major transcription start site. A second region (MNII), with a partially increased sensitivity in wild-type animals, was located around −800 bp. The two cleavage sites at MNI were approximately 200 bp apart and might be separated by a single positioned nucleosome or nucleoprotein complex. Its position coincides with the DNase I-hypersensitive site HSSII, where two HNF1 binding sites have been identified. When the same micrococcal nuclease sensitivity assay was performed on nuclei from HNF1α-deficient livers, MNI was undetectable and MNII was much less prominent. These observations suggest that HNF1 binding could be responsible for the altered micrococcal sensitivity of the locus. Similarly, the HSSIII region showed a digestion pattern that differed between mutant livers and the controls (data not shown).

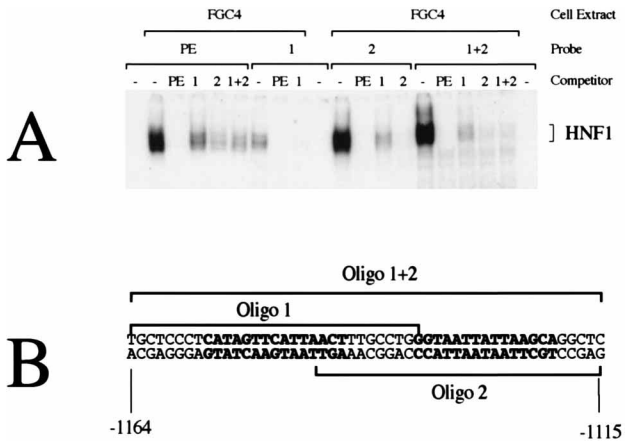

FIG. 4. Gel mobility shift analysis with HNF1 binding sites in the PAH HSSII region. (A) Labeled double-stranded oligonucleotides (0.4 ng) were incubated with nuclear extracts from differentiated rat hepatoma FGC4 cells in the presence or absence of a 50-fold excess of unlabeled oligonucleotide competitor as indicated. HNF1 complexes are indicated by a bracket at the right. (B) Sequence of the region containing the HNF1 binding sites located between 1,156 and 1,120 bp upstream of the most 5′ transcriptional start site of the PAH promoter. The sequence showing homology to the HNF1 consensus is shown in boldface type. The sequence of the oligonucleotides used for the gel mobility assay is indicated.

The cleavage patterns observed in HNF1α-deficient livers or in the spleens of normal animals closely resembled the cleavage pattern obtained with free DNA. Hence, this cleavage pattern was probably dictated only by the sequence-specific preferential cleavage of micrococcal nuclease. This observation suggests that in nuclei where HNF1 is not present (spleen or HNF1α-deficient liver) the nucleosomes located on this region of the PAH promoter are randomly positioned. At least some of them may be displaced or modified in normal liver in an HNF1-dependent manner.

Hepatic but not renal PAH expression is affected in HNF1α-deficient animals. In contrast to human PAH, which is expressed exclusively in the liver, murine and rat PAH are also expressed at low levels in kidney proximal tubules (49). As mentioned above, PAH expression in HNF1α-deficient mice is drastically compromised in the liver. In this organ, HNF1α is

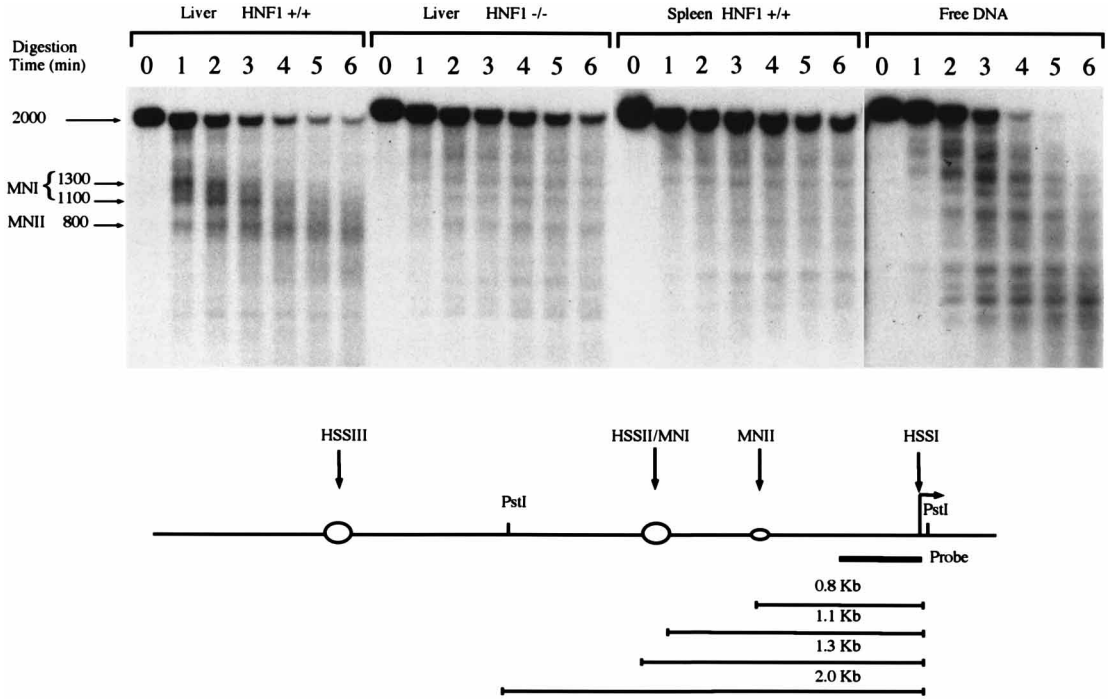

FIG. 5. Tissue-specific micrococcal nuclease sensitivity over the PAH promoter-enhancer region. (Above) Nuclei from mouse liver, spleen, and protein-free DNA (free DNA) were treated with micrococcal nuclease for increasing times as indicated. The DNA was digested with *Pst*I and subjected to Southern blot analysis with the probe indicated in the bottom scheme. (Below) Summary of micrococcal nuclease-hypersensitive (MN) and DNase I-hypersensitive (HSS) sites on the PAH promoter-enhancer. The position of the probe is indicated by the thick bar. The length of the *Pst*I fragment and the micrococcal nuclease digestion products hybridizing with the probe are indicated below.

normally relatively abundant, and its level is roughly 20 times higher than that of HNF1β. In contrast, in the kidneys, HNF1α and HNF1β are expressed at roughly equal levels.

The residual abundance of HNF1β protein in the kidneys of HNF1α-deficient animals and the persistence of specific DNase I hypersensitivity prompted us to examine whether PAH expression in this organ was affected by the inactivation of HNF1α. Northern blots of total RNA from the kidneys showed that renal PAH was expressed at the same level in HNF1α-deficient animals and their controls (data not shown). In agreement with the results of DNase I-hypersensitive-site analysis (Fig. 3), the nucleosomal position, as revealed by micrococcal nuclease digestion, did not show any significant difference between the kidneys of HNF1α-deficient animals and the controls (data not shown). To determine whether HNF1β could functionally replace HNF1α in the PAH transcriptional activation in the kidneys, we compared the levels of HNF1α and HNF1β in the livers and kidneys from wild-type and mutant animals. The results, shown in Fig. 6, demonstrate that the f3 HNF1 binding site localized in the HSIII enhancer is bound by both HNF1α and HNF1β very avidly in band shift assays. It is noteworthy that the level of HNF1α protein in the livers of wild-type animals is comparable to the level of HNF1β in the kidneys of mutant mice. In addition, cotransfection experiments indicated that HNF1β can transactivate, as efficiently as HNF1α, a reporter plasmid carrying three copies of the f3 site upstream of a minimal herpes simplex virus thymidine kinase promoter (data not shown). These results indicate that HNF1β should be able to provide a functional replacement for HNF1α in the kidneys. However, we cannot rule out that the PAH gene is regulated by a different combination of factors in the kidney, with HNF1α playing a subsidiary role.

HNF1α-deficient animals suffered from a severe hyperphenylalaninemia notwithstanding the normal renal PAH expression. This observation reinforces the idea that under normal conditions, the conversion of phenylalanine to tyrosine is carried out mainly in the liver and that the renal contribution alone is not sufficient to guarantee this task.

PAH transcriptional inactivity is associated with methylation of the regulatory region. It has been shown that DNA hypermethylation is tightly correlated with the transcriptional inactivity of a gene. DNA methylation normally occurs at specific CpG residues located close to the regulatory elements of inactive genes. Methylation affects nonhousekeeping genes and is usually set at the time of implantation of the embryo.

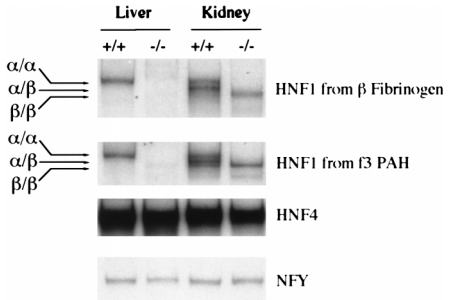

FIG. 6. HNF1α and β DNA binding activities. Nuclear extracts (3 μg per lane) from liver and kidney from either wild-type or HNF1α-deficient animals were used for a bandshift assay with the probes indicated to the right of the figure. "HNF1 from β Fibrinogen" stands for the rat β-fibrinogen HNF1 binding site (16). "HNF1 from f3 PAH" stands for the f3 element located in the HSIII enhancer of the PAH transcriptional control regions (22). HNF4 corresponds to the mouse HNF1α promoter HNF4 binding site. NFY stands for the albumin-proximal-promoter CAAT binding site.

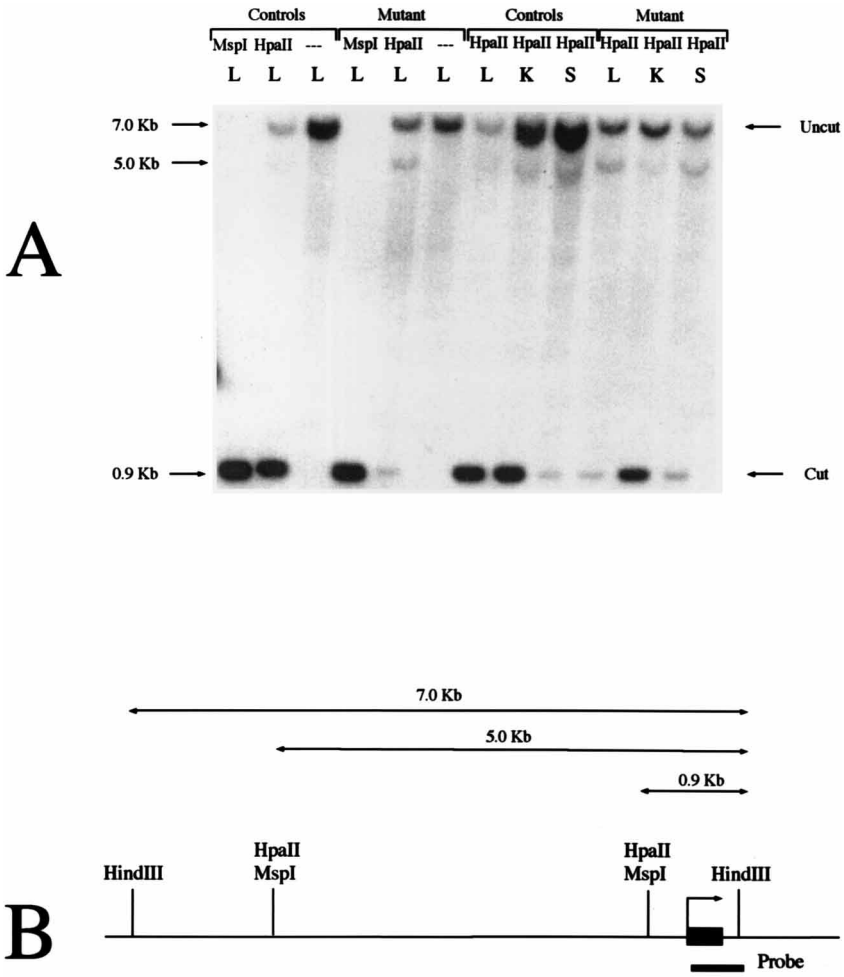

FIG. 7. Methylation status of a *Hpa*II site in the PAH regulatory region. (A) Genomic DNA from liver (L), kidneys (K), or spleen (S) from control or from HNF1α-deficient animals (Mutant), after digestion with *Hin*dIII, was cleaved with either *Msp*I or *Hpa*II as indicated. DNA was analyzed by Southern blotting with a probe abutting the *Hin*dIII site (B). The positions of the methylated and nonmethylated fragments are indicated by arrows on the right. (B) Localization of the *Hpa*II and *Msp*I sites in the PAH enhancer-promoter. The length of the fragments derived from the cleavage with *Hpa*II is indicated.

During the differentiation process, the genes that become active undergo a specific programmed demethylation that affects only the cell types in which the gene is activated.

We examined whether the lack of hepatic PAH expression was associated with a different degree of methylation of the PAH promoter in HNF1α−/− animals. To this end, we monitored the *Hpa*II cleavage efficiency at the only site in the region, which is located roughly 400 bp upstream of the transcription initiation site (Fig. 7). *Hpa*II digestion of genomic DNA from livers of control animals showed a strong band corresponding to cleaved DNA at this *Hpa*II site, implying that this site is predominantly unmethylated. In contrast, DNA from HNF1α-deficient livers gave a barely visible band, suggesting that HNF1-deficient animals have a strong degree of methylation at this site (Fig. 7). This degree of methylation was comparable to that observed in the spleen. There was no significant difference in the degree of methylation in the kidneys of heterozygotes and mutant animals. The appearance of an additional band of approximately 5 kb suggests the presence of a second *Hpa*II site, whose degree of methylation has not been characterized in detail.

Since albumin expression was reduced but not abolished in the mutant animals, we wondered if hypermethylation also occurred in the albumin promoter. *Hpa*II cleavage showed that a site located 76 bp downstream of the transcriptional start site was undermethylated in the mutant mice although to a lesser extent than in control animals (data not shown). This milder effect of HNF1α inactivation on the degree of methylation of the albumin promoter indicates that HNF1α does not play a major role in the developmental demethylation of this gene.

DISCUSSION

The analysis, by transfection or in vitro transcription studies, of *cis*-acting elements found in the promoters of liver-specific genes has led to the characterization of HNF1α as one of the most prominent liver-enriched transacting factors. HNF1α was shown to activate the transcription of many hepatic genes including plasma proteins, enzymes, and even hepatitis B viruses (reviewed in reference 44). Gene targeting provides novel opportunities for understanding the mechanisms and the functions involved in transcriptional activation. Disruption of the HNF1α gene confirmed its role in the transcriptional activation of previously identified target genes. However, these studies also raised further questions. While expression of genes like the albumin and α_1-antitrypsin genes was reduced only mod-

erately by two- to fourfold, PAH gene expression was drastically compromised. The complete failure to activate this gene, compared to the much milder effects of the deficiency on other HNF1 targets, was enigmatic. In the present study, we have attempted to clarify the mechanisms through which the inactivation of a single transcription factor results in the lack of expression of a specific target gene whose expression depends upon multiple factors.

Our results suggest that the presence of HNF1α is essential for chromatin remodeling and demethylation of the transcription control sequences of the PAH gene, presumably a prerequisite for transcriptional activation. In fact, all of the tissue-specific DNase I-hypersensitive sites we monitored in the PAH promoter/enhancer region (HSSI, HSSII, and HSSIII) were undetectable in liver nuclei from HNF1α-deficient animals. It has been shown that HSSII and HSSIII (22) contain HNF1 binding sites. It cannot be ruled out that DNase I hypersensitivity, at least in part, was the direct consequence of the HNF1 binding to these elements. It is known that the binding of a protein to chromatin can provoke DNA distortion that can be responsible for an increased DNase I sensitivity. However, one has to recall that HNF1α is not the sole DNA binding protein that interacts with the HSIII enhancer (22). In addition, the use of micrococcal nuclease revealed that a series of liver-specific cleavage sites extending for 400 to 500 bp and including HSSII and HSSIII were absent in nuclei from mutant animals. Micrococcal nuclease is known to cleave specifically the internucleosomal DNA linkers or the DNA that is wrapped around modified nucleosomal structures. Hence, our observations suggest that HNF1 may somehow induce chromatin remodeling involving the displacement or modification of nucleosomes over a region of 400 to 500 bp, a region that is much larger than that spanned by the HNF1 binding sites.

We have also observed that the PAH promoter was methylated in HNF1α-deficient livers but not in control animals. DNA hypermethylation has been shown to play a causative role in determining the chromatin structure of a gene (9, 27, 50). In the case of the TAT promoter, hypermethylation is associated with chromatin structures that prevent the interaction of transcription factors with their target sites in fibroblasts that do not express the gene (4). The notion that DNA methylation plays a key role in the regulation of mammalian genes is further supported by the observation that methyltransferase gene inactivation results in an embryonic lethal phenotype (31). It is known that transcription factor binding sites can protect DNA from de novo methylation; in addition, demethylation is not necessarily linked to ongoing transcription (8). For example an Sp1 binding site protects neighboring sequences from methylation, although the nature of the proteins that bind to this Sp1 element is not clear. Our study shows that a transcriptional defect caused by the deletion of the HNF1α transcription factor was directly correlated with a change in the methylation and chromatin state of the PAH regulatory region. Further experiments are essential to establish whether the methylation is the cause or simply the consequence of the lack of transcription of a gene.

Hormonal treatment failed to restore the activation of the PAH promoter in mutant animals, suggesting that HNF1α disruption has rendered the gene insensitive to the hormones. It is reasonable to postulate that in the absence of chromatin opening the hormone receptors are prevented from interacting with the HSIII enhancer in a productive way. The normal inducibility of TAT expression observed in HNF1α-deficient animals demonstrates that the signal transduction pathways mediating hormone response were not compromised by the inactivation of HNF1α.

Two of the DNase I-hypersensitive sites in the PAH regulatory region (HSSII and HSSIII) contain HNF1 binding sites. Mutation of these sites in the HSIII enhancer has little effect on the basal activity of the enhancer but does severely compromise hormone responsiveness, implying a collaboration between HNF1α and hormone receptors for transcriptional activity (22). However, since hormone inducibility of genes expressed specifically in the liver is primarily a postnatal response, while establishment of chromatin domains occurs during embryogenesis, our attention was focused upon HSSII.

Deletion studies show that HSSII does not seem to play any significant role in transient-transfection assays (22). We now show that this region contains two bona fide HNF1 binding sites, which show the unusual property of being separated by only 7 nucleotides. Such proximity of HNF1 binding sites is a configuration that has not been observed for other genes (42). We propose that this pair of HNF1 sites could be important for chromatin remodeling.

At least two distinct mechanisms can be evoked for the proposed chromatin-opening function of the HSSII region with its HNF1 sites. Even though this element lacks enhancer activity in transient transfections, it could induce a low level of transcriptional activation in the presence of HNF1α in the embryonic liver. This initial transcriptionally active status could function to stabilize an open chromatin structure. This particular chromatin configuration could then make the regulatory regions responsive to the strong hormonal stimuli at birth. In this model, HSSII would behave as an enhancer that is active specifically during embryogenesis.

An alternative hypothesis would propose for HSSII a more direct role in the establishment of open chromatin domains possibly through a cooperative interaction with HSSIII. There are several precedents of the existence of such chromatin-modifying elements. The activity of HSSII is reminiscent of the effects operated by the HS3 site of the human β-globin locus control region (LCR) in transgenic mice (21). In this case, expression of a single-copy integrated transgene can be guaranteed by the presence of a combination of two DNase I-hypersensitive sites called HS2 and HS3. While HS2 is the sole element that fulfills the classical definition of enhancer, its full activity is conditioned by the presence of HS3. This second element is responsible for the chromatin transitions observed in hematopoietic cell lineages. The LCR effect could be the result of two combined functions: a recessive enhancer activity exerted by HS2 and a chromatin-remodeling activity exerted by HS3. The deletion of certain hypersensitive sites in the LCR has severe effects on transcription without affecting the global DNase I hypersensitivity at other positions in the locus (35). Hence, transcriptionally inactive genes are not necessarily in a "closed" chromatin configuration and the extent of DNase I hypersensitivity is not proportionally linked to transcription. These notions are further supported by the observation for HNF1α−/− animals that the overall albumin promoter DNase I hypersensitivity is not grossly changed in spite of a fourfold reduction of the transcription rate of the gene (data not shown). Taken together, these considerations suggest that the "closed" PAH gene configuration in HNF1α-deficient animals is not simply a consequence of the silent transcriptional status of the gene. On the contrary, this "closed" chromatin structure could be responsible for the transcriptionally inactive status of the PAH gene. It seems unlikely that binding of HNF1 alone is sufficient for such an opening activity. Indeed, very strong HNF1 binding sites are present in genes like immunoglobulin or β globin genes that are DNase I resistant in hepatocytes (42). In this respect, HNF1α may be dissimilar from the GAGA (45) or Pho5 (40) factors, which can directly induce

disruption of nucleosomes. HNF1α could function in conjunction with other proteins binding to HSSII. Further studies are required to define the partners of HNF1 and the mode of action of HSSII and HSSIII in vivo.

The data presented here indicate that HNF1α could accomplish its transactivation tasks through two distinct activities: the first is the direct transcriptional activation by recruitment of the transcription machinery to the promoter, and the second would concern the establishment of an "open" chromatin configuration, which also involves DNA demethylation, on promoter-enhancer sequences. These chromatin modifications would permit subsequent productive interaction of transcription factors such as the hormone receptors with their cognate sequences. Other transcription factors may play such a dual role. Their inactivation will more drastically affect genes where they play a major role in chromatin remodeling and demethylation than genes where they participate only in combination with other factors in recruitment of the general transcriptional machinery. The phenotype of mutant mice lacking a specific transcription factor may depend more on the nature of the genes that are totally shut off than on target genes that are transcribed at a reduced rate.

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