Deficiency of an enzyme of tyrosine metabolism underlies altered gene expression in newborn liver of lethal albino mice

Siegfried Ruppert,1,2 Gavin Kelsey,2 Andreas Schedl, Erika Schmid, Edda Thies, and Günther Schütz3

Institute of Cell and Tumor Biology, German Cancer Research Center, W-6900 Heidelberg, Germany

Mice homozygous for albino deletions encompassing the locus alf/hsdr-1 die shortly after birth. Lethality is thought to be the consequence of hypoglycemia, which results from the failure to activate hormone-dependent genes in liver and kidney encoding enzymes important for gluconeogenesis. Within the region in which alf/hsdr-1 has been defined by physical mapping, we identified the gene encoding fumarylacetoacetate hydrase (FAH), an enzyme of tyrosine metabolism. Lack of FAH activity should lead to accumulation of toxic tyrosine metabolites. In man, genetically determined FAH deficiency is the primary defect in tyrosinemia type I, a fatal liver disease of infants. Northern blot and in situ hybridization analysis of mouse tissues showed that the cell types that normally express FAH correspond to those that exhibit a phenotype in alf/hsdr-1 deletion mice. Moreover, we could mimic aspects of the alf/hsdr-1 deletion phenotype in vitro by treating primary hepatocyte cultures with an intermediate of tyrosine metabolism. These findings strongly suggest that alf/hsdr-1 encodes FAH and that absence of FAH is responsible for neonatal lethality in albino deletion mice. Mechanisms by which this metabolic defect might bring about alterations in gene expression characteristic of the alf/hsdr-1 deletion phenotype are discussed.

[key Words: alf/hsdr-1; albino-deletion complex; fumarylacetoacetate hydrase; liver-specific gene expression; hormone inducibility; tyrosinemia type I]

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Mutations that affect the differentiation of specific cell types in higher eukaryotes have enormous potential for identifying factors that contribute to cell type-specific gene expression. One such genetic resource in the mouse is the albino-deletion complex on chromosome 7, which comprises at least 37 radiation-induced chromosomal deletions overlapping at the albino locus, c. The deletions have been organized into 13 complementation groups, which define several loci essential for normal development before or after birth [Russell et al. 1982; for review, see Rinchik and Russell 1990]. One locus is necessary for survival beyond birth and has been proposed to play an important role in the perinatal differentiation of the hepatocyte [Gluecksohn-Waelsch 1979]. The locus is designated alf [factor indicated by the albino lethal mutation [Ruppert et al. 1990]] or hsd-1 [hepatocyte-specific developmental regulation-1 [McKnight et al. 1989]] and is referred to as alf/hsdr-1 in this paper.

Mice homozygous for albino deletions that include alf/hsdr-1 die several hours after birth. Lethality is associated with severe hypoglycemia, and the failure to activate several enzymes in liver involved in gluconeogenesis, notably glucose-6-phosphatase, tyrosine aminotransferase (TAT), and phosphoenolpyruvate carboxykinase [PEPCK] [Erickson et al. 1968; Thorndike et al. 1973; Schmid et al. 1985; Loose et al. 1986]. Many other enzymes and proteins are present at normal levels [Gluecksohn-Waelsch 1979; Baier et al. 1984]. Decreased transcription has been shown to be responsible for these and other enzyme deficiencies [Loose et al. 1986; Morris et al. 1988; Ruppert et al. 1990]. Analysis of a representative set of affected mRNAs identified by subtractive cDNA hybridization suggested that expression of the affected genes was normally inducible by glucocorticoids and/or cAMP around birth in liver [Ruppert et al. 1990]. In contrast, expression of glucose-6-phosphatase, TAT, and the metallothionein Mt-1 gene cannot be induced by exogenous hormones in mutant liver [Thorndike et al. 1973; Schmid et al. 1985; DeFranco et al. 1988]. These observations were compatible with a hypothesis that alf/hsdr-1 encodes a regulatory factor essential for cell type-
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specific and hormone-dependent gene expression (Gluecksohn-Waelsch 1987; Ruppert et al. 1990). A specific deficit of factors in the signal transduction pathways for glucocorticoid and cAMP has not been found, however (Ruppert et al. 1990; DeFranco et al. 1991). In contrast, the mRNAs for some transcription factors enriched in liver, C/EBP, HNF-1, and HNF-4, are present at reduced levels (McKnight et al. 1989; Gonzalez et al. 1990; Ruppert et al. 1990; Tönjes et al. 1992).

The reduced expression of specific mRNAs in the affected mice is limited to two cell types: the parenchymal cells of the liver, and proximal convoluted tubule cells in the kidney (Ruppert et al. 1990). Analysis by electron microscopy has revealed that extensive ultrastructural abnormalities are present in the same two cell types in late fetal and newborn mutant mice (Trigg and Gluecksohn-Waelsch 1973).

Whereas much interest has focused on decreased expression of liver-specific genes, some mRNAs show increased abundance in livers of mice homozygous for neonatally lethal albino deletions. These include the mRNA for NAD(P)H : menadione oxidoreductase (NMO-1), an enzyme involved in oxidative detoxification (Petersen et al. 1989), and three mRNAs induced by growth arrest and DNA damage, so-called gadd transcripts (Fornace et al. 1989). This adds to earlier findings of increased UDP-glucoronyltransferase and glutathione S-transferase B (GST) activities (Thaler et al. 1976; Gatmaitan et al. 1977), two enzymes which, like NMO-1, belong to the aryl hydrocarbon (Ah) battery (Nebert et al. 1990). A locus encoding a negative regulator of members of the Ah battery, designated Nmo-In, has been proposed from these observations (Nebert et al. 1990).

Because of the pleiotropic phenotype associated with neonatally lethal albino deletions, the function of the alf/hsdr-1 gene product has been difficult to predict. Therefore, we sought to isolate alf/hsdr-1 by "positional cloning." We established a physical map of the region of chromosome 7 corresponding to the albino-deletion complex including the ~3800 kb absent in the "prototypic deletion" c°4c°s (Kelsey et al. 1992). From markers closely flanking alf/hsdr-1, chromosome jumping libraries were then used to isolate new markers mapping between the proximal breakpoints of the deletions c°7sM and c°1DsD, the deletions that bracket alf/hsdr-1 (Schedl et al. 1992). The identification of these two breakpoints allowed us to limit alf/hsdr-1 to ~310 kb, and more than one-third of this region has now been cloned by walking in λ and cosmid libraries (Schedl et al. 1992).

We set the following criteria that a candidate gene for alf/hsdr-1 should satisfy: [1] the gene must be entirely or partly absent from c-locus deletions that involve alf/hsdr-1; [2] assuming that the gene product acts cell autonomously, the gene must be expressed in hepatocytes and in proximal tubular cells of the kidney; and [3] the gene must normally be expressed at a time preceding or coinciding with the development of the alf/hsdr-1 deletion phenotype.

We identified the gene for fumarylacetoacetate hydrodase [FAH] within the alf/hsdr-1 region. Identification of the Fah gene suggests a novel and unifying explanation for the alf/hsdr-1 deletion phenotype.

Results

The alf/hsdr-1 deletion phenotype develops before birth in liver and kidney

The altered gene expression in mice homozygous for deletions including alf/hsdr-1 has been analyzed most extensively in newborn liver, and it has been concluded that expression of TAT and PEPCK is affected only after birth (Donner et al. 1988). To determine when a phenotype first arises in mice homozygous for the alf/hsdr-1 deletion c°4c°s, we followed the expression of a set of mRNAs in liver before and after birth. The mRNAs included those for TAT, PEPCK, S-adenosylmethionine synthetase (SAMS), mitochondrial 3-hydroxy-3-methylglutaryl-CoA-synthase (CoAS), and an anonymous transcript, X1, whose cDNA was among those identified by differential screening (Ruppert et al. 1990). PEPCK mRNA was not detected before birth, and a decreased abundance in mutant liver is first apparent immediately after birth when PEPCK expression is strongly induced in wild-type mice (Fig. 1). The low levels of TAT and X1 mRNAs present before birth are only slightly reduced, and the failure to attain high levels after birth is the
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The greatest effect in mutant liver. SAMS and CoAS mRNAs are readily detectable before birth and are present in similar amounts in mutant and wild-type livers at day 16.5 of gestation. Subsequently, their levels decline in mutant liver, and SAMS fails to exhibit the strong neonatal induction characteristic of wild type. That decreases in the steady-state level of some mRNAs do occur before birth is in agreement with the recent observation of reduced transcription rates for several genes in late fetal c14CoS/c14CoS liver (Tönjes et al. 1992).

The mRNA for the detoxifying enzyme NMO-1 is highly induced in c14CoS/c14CoS liver (Peterson et al. 1989). We find that NMO-1 mRNA is present at a high level at day 16.5 both in mutant liver and kidney [Fig. 1]. This is the earliest aspect of the c14CoS/c14CoS phenotype detected. We also examined the expression of FOS mRNA to extend a previous finding of elevated FOS mRNA in newborn liver of mutant mice (Ruppert 1988). The level of FOS mRNA was low in liver of wild-type mice, except for an increase around the first hour after birth. The expression profile in c14CoS/c14CoS liver differed strikingly in two respects: FOS mRNA was clearly increased in newborn liver of mutant mice (Ruppert 1988). This is the earliest aspect of the deletion phenotype. By day 16.5, FOS mRNA is elevated at fetal day 18.5, and a high level is maintained for several hours after birth [Fig. 1].

**Figure 2.** The alf/hsdr-1 deletion phenotype results from the loss of a gene(s) located in a ~315-kb interval. (A) Schematic representation of the location of alf/hsdr-1 on mouse chromosome 7. (Top line) BssHII. (B) restriction sites on the wild-type chromosome mapped within the region defined by the albino deletion complex (Kelsey et al. 1992). (c) Albino locus, (c) the centromere. Below, the proximal extensions of the c14CoS, c10R75M, and c11DSD deletions are shown by open arrows according to Schedl et al. (1992). All or part of a candidate gene for alf/hsdr-1 maps within the ~315-kb interval between the c11DSD and c14CoS deletion breakpoints, the broken arrow indicates the possibility that a candidate gene might extend beyond the proximal extremes of the c10R75M or c14CoS deletions. The proximal breakpoints of the c10R75M and c14CoS deletions are separated by 5 kb (Schedl et al. 1992). alf/hsdr-1 is ~3000 kb from c (Kelsey et al. 1992). (B) Total RNA (5 μg) isolated shortly after birth from the livers of lethal albino mice [c14CoS/c14CoS], and their normal counterparts [c10R75M/c10R75M or c11DSD/c11DSD]. The mRNA for the detoxifying enzyme NMO-1 is present at a high level in normal liver and undetectable in newborn liver of mutant mice. SAMS and CoAS mRNAs are severely reduced in c14CoS homozygotes, they are present in normal amounts in the c11DSD/c14CoS double heterozygote (Fig. 2B). In addition, the elevation of NMO-1 mRNA was not seen in c11DSD/c14CoS liver. Therefore, all facets of the c14CoS deletion phenotype so far examined depend on the loss of a gene(s) that maps to the same ~315-kb interval between the proximal breakpoints of c14CoS and c11DSD deletions.

A gene identified within the alf/hsdr-1 region encodes FAH

Nearly one-third of the region between the proximal boundaries of the c14CoS and c11DSD deletions has been isolated as λ and cosmid contigs (Schedl et al. 1992). To identify potentially expressed sequences, single-copy probes from each contig were tested for cross-species ho-
mology by hybridization to "zoo blots" (data not shown). Cloned regions proximal to the \( c^{14C_{0S}} \) deletion were also screened because of the possibility that a candidate gene could extend beyond the proximal extreme of the deletion. Three probes [RN.Fd, RN.Fa, and JRN2.3 in Fig. 4B, below] in a 10-kb region of a contig designated RN (for the location of this contig, see Schedl et al. 1992) spanning the proximal end of the \( c^{14C_{0S}} \) deletion detected cross-hybridization with rat, human, and bovine DNAs (data not shown). In addition, the same probes hybridized on Northern blots to a ~1.6-kb transcript in RNA from newborn livers of wild-type but not \( c^{14C_{0S}/C^{14C_{0S}}} \) mice (data not shown). These probes were used to screen a newborn liver cDNA library, and 12 cDNA clones were isolated. Four near full-length cDNAs were sequenced completely and found to encode a polypeptide of 419-amino-acid residues with 88% amino acid sequence identity to human FAH (Fig. 3A), whose cDNA was isolated recently [Agsteribbe et al. 1990; Phaneuf et al. 1991]. FAH catalyzes the final reaction in the degradation of tyrosine [Fig. 3B]. FAH deficiency in man is the primary defect in the autosomal recessive liver disease tyrosinemia type I [Kvittingen 1986], whose significance for the \( alf/hsdr-1 \) deletion phenotype is dealt with in the discussion.

The three genomic fragments that identified the FAH transcript overlap the proximal border of the \( c^{14C_{0S}} \) deletion and are proximal to the \( c^{10R75M} \) deletion [Fig. 4B]. Because these two c-locus deletions have breakpoints that are closest proximally to \( alf/hsdr-1 \), we investigated how the Fah gene was altered by the deletions. Homozygous \( c^{10R75M} \) and \( c^{14C_{0S}} \) DNAs were compared with wild-type control \( c^{Ch/c_{ch}} \) and \( C^{3H} \) DNAs on Southern blots hybridized with a near full-length \( Fah \) cDNA, pm-cFAH3 (Fig. 4A). Altered restriction fragments were detected in both deletion DNAs, from which it was concluded that the \( c^{14C_{0S}} \) deletion removes the first two exons of the \( Fah \) gene while the \( c^{10R75M} \) deletion does not disrupt coding regions, but its proximal breakpoint maps <1 kb 5' to the start site of transcription [Fig. 4B; the structural characterization of the \( Fah \) gene will be presented elsewhere (G. Kelsey, unpubl.)]. FAH mRNA is not detected in newborn liver of \( c^{14C_{0S}} \) homozygotes.

Figure 3. A gene identified within the \( alf/hsdr-1 \) region encodes FAH. (A) Amino acid comparison [shown as the single-letter code] of the derived amino acid sequence from the full-length cDNA clone pmcFAH7 with that of human FAH [Agsteribbe et al. 1990; Phaneuf et al. 1991]. (Top) The mouse sequence; (bottom) identical amino acid residues between the mouse and human sequences are indicated by a dash in the human sequence. Numbers at left refer to amino acid residues from the presumed initiating methionine. (B) Representation of the tyrosine degradation pathway. The reaction catalyzed by FAH is enclosed within the box.
Figure 4. Location of the c14CoS and c10R75M deletion breakpoints within the mouse Fah gene. (A) Southern blots of EcoRI, HindIII, XbaI and BamHI-digested DNAs from mice homozygous for the c14CoS or c10R75M deletions and control homozygous ch and C3H mice were hybridized with a near full-length FAH cDNA, pmcFAH3. pmcFAH3 lacks sequences corresponding to exon 13 of the Fah gene and was used to avoid detection of exon 13 containing EcoRI and HindIII fragments, which comigrate with other exon fragments [G. Kelsey, unpubl.]. The numbers adjacent to the ch lanes refer to the exons present on the indicated restriction fragments. The arrows identify fragments of altered mobility in the two deletion DNAs. An EcoRI site absent on the ch allele (identified by an asterisk in B) gives rise to a larger fragment for exons 11-13 [shown as (11-13)]. (©) An EcoRI fragment in the c14CoS lane suspected to be a partial digestion product. (B) The extents of the c14CoS and c10R75M deletions are shown as solid arrows below the map of EcoRI, HindIII, XbaI, and BamHI restriction sites of the mouse Fah gene. The c10R75M deletion breakpoint has been mapped between BamHI and HindIII sites within genomic fragment JRN2.3 [Schedl et al. 1992]. Exons of the Fah gene are represented by open boxes. Derivation of the exon/intron organization will be presented elsewhere [G. Kelsey, unpubl.]. The three single-copy genomic fragments that showed cross-hybridization between mouse, human, and calf DNA and that identified the FAH transcript in liver RNA are indicated by the stippled boxes. The EcoRI site absent on the ch allele is marked by an asterisk (*).

(Fig. 5B) nor c10R75M homozygotes [Klebig et al. 1992]. The failure of the Fah gene to be expressed from the c10R75M chromosome might indicate that the deletion removes sequences essential for expression. Consistent with this notion, we have found that a region underlying a DNase I hypersensitive site in liver and kidney chromatin associated with Fah gene activity is removed by the deletion [G. Kelsey, unpubl.].

The Fah gene is expressed before birth and in cell types that display the alf/hsdr-1 deletion phenotype

The expression of FAH mRNA was investigated to see whether it satisfied expectations for a candidate gene for alf/hsdr-1. The alf/hsdr-1 deletion phenotype is present specifically in liver and kidney. Northern blot analysis of RNAs from adult tissues revealed high-level expression
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Figure 5. Expression analysis of the Fah gene by Northern blot hybridization. (A) The tissue specificity of FAH mRNA expression was analyzed with FAH cDNA pmcFAH3. Total RNAs were from adult mouse tissues, except for hypothalamus, adrenal gland, and ovary, which were from rat. On longer exposures of the autoradiograph, low levels of FAH mRNA are detected in all lanes. In B, C, and D, Northern blots identical to those shown in Figs. 1 and 2 were hybridized with pmcFAH3.

of FAH mRNA in the liver and kidney, whereas very much lower levels were present in all remaining tissues (Fig. 5A). A phenotype develops in c^{14}CoS homozygotes by day 16.5 of gestation (e.g., elevated NMO-1; Fig. 1), and we find that FAH mRNA is expressed as early as fetal day 16.5 in both liver and kidney in wild-type mice (Fig. 5B,C). Finally, although no FAH transcripts were detected in c^{14}CoS liver, FAH mRNA expression was similar to wild type in newborn liver of the complemented heterozygote c^{11}SDS/c^{14}CoS (Fig. 5D).

Because the effects on gene expression and the ultrastructural lesions are restricted to parenchymal cells of the liver and proximal convoluted tubule cells in the kidney (Trigg and Gluecksohn-Waelsch 1973; Ruppert et al. 1990), in situ hybridization was performed to identify the cell types in which FAH mRNA is normally present. Sections of liver and kidney from late fetal and newborn wild-type and c^{14}CoS/c^{14}CoS mice were hybridized with antisense probes for FAH, PEPCK, and NMO-1. Hybridizations of kidney sections are shown in Figure 6. In wild-type kidney, PEPCK and FAH hybridization is detected predominantly in proximal tubules (Fig. 6A–D). There was no specific hybridization of the FAH probe to c^{14}CoS/c^{14}CoS kidney (Fig. 6E). NMO-1 expression was detected only in c^{14}CoS/c^{14}CoS kidney (Fig. 6, F and G), and hybridization was also localized to proximal tubule cells (Fig. 6, G and H). In wild-type liver, PEPCK and FAH hybridization was confined to hepatocytes, and NMO-1 expression was restricted to c^{14}CoS/c^{14}CoS hepatocytes [data not shown]. Together, these results show a tight correlation between the normal sites and timing of FAH expression and cells displaying the alf/hsdr-1 deletion phenotype.

Expression of the alf/hsdr-1 deletion phenotype in primary hepatocytes

Because FAH catalyzes the final step in the breakdown of tyrosine, its deficiency may result in the accumulation of tyrosine metabolites, in particular, maleylacetoacetate [MAA] and fumarylacetoacetate [FAA] (Fig. 3B). These are considered to be the reactive intermediates responsible for initiating the liver damage that accompanies Fah deficiency in man (Kvittingen 1986). The identification that the Fah gene is disrupted by the c^{14}CoS deletion raised the possibility that the alterations in gene expression characteristic of the alf/hsdr-1 deletion phenotype are the secondary consequences of toxic effects of FAA and/or MAA. To obtain an indication for this, we attempted to reproduce some aspects of the phenotype in vitro by influencing tyrosine metabolism in cultured hepatocytes.

First, primary hepatocytes were examined for the expression and hormone inducibility of the mRNAs affected by deletion of alf/hsdr-1. Livers were removed from day 17.5 or 18.5 c^{14}CoS/c^{14}CoS (albino) and wild-type (pigmented) fetuses, and cultures of partially enriched hepatocytes were treated with glucocorticoid (dexamethasone) or a cAMP analog (CPT-cAMP) and RNA was prepared. TAT mRNA was not detected in the absence of hormones. However, in both wild-type and c^{14}CoS/c^{14}CoS hepatocyte cultures, TAT mRNA accumu-
Figure 6. [See facing page for legend.]
lated in response to dexamethasone and CPT-cAMP and
a combination of the two synergistically induced TAT
mRNA to similar high levels in both cultures [Fig. 7A]. The mRNA for SAMS behaved in a similar way [Fig. 7A]. The origin of the cultures could be verified by detection of TAT and SAMS mRNAs (Fig. 7B, and data not shown). HGA treatment (500 μM) was found to reduce TAT and SAMS mRNAs in cultures induced with dexamethasone and forskolin close to uninduced levels, both in wild-type and c14Cos/c14Cos cells [Fig. 7B]. Hybridization with a probe for transferrin indicates that there was no general effect of HGA on mRNA levels. NMO-1 mRNA accumulated in response to HGA treatment, with c14Cos/c14Cos hepatocytes displaying an apparently greater increase [Fig. 7B]. A differential response in NMO-1 expression between wild-type and c14Cos/c14Cos hepatocytes was seen more clearly in a separate experiment in which different concentrations of HGA were added at the same time as the hormone inducers. In this experiment there was a less pronounced effect of HGA treatment on TAT and SAMS mRNAs [Fig. 7C, and data not shown]. HGA induced NMO-1 mRNA in a dose-dependent manner, with a particularly strong increase in c14Cos/c14Cos hepatocytes treated with 500 μM HGA [Fig. 7C]. The increase in NMO-1 mRNA was slightly augmented by hormone treatment, and a significant hormone induction of NMO-1 mRNA was detected in c14Cos/c14Cos hepatocytes even in the absence of HGA. A stronger effect on

Figure 7. Expression of the alf/hsd1 deletion phenotype in primary hepatocytes. (A) Primary hepatocytes from wild-type and lethal albino (c14Cos/c14Cos) mice were isolated from day 18 embryos. After 2 days in culture, cells were induced with dexamethasone (Dex), CPT-cAMP (cAMP), dexamethasone plus CPT-cAMP, or mock-induced. Dexamethasone induction was for 16 hr, CPT-cAMP for 6 hr. Cells were harvested, and Northern analysis was performed on 10 μg of total RNA, with the cDNA probes indicated. Hybridization with a transferrin cDNA probe is shown (Tf). (B) Primary hepatocytes from albino lethal (c14Cos/c14Cos) and wild-type mice were treated with HGA (500 μM) in the presence or absence of dexamethasone and forskolin, which increases cAMP levels after 2 days in culture. Cultures were exposed to dexamethasone plus forskolin for the final 23 hr and HGA for the final 17 hr before harvesting. Four micrograms of total RNA was hybridized to the indicated probes. (C) Primary hepatocytes from lethal albino (c14Cos/c14Cos) and wild-type mice were treated with 100 or 500 μM HGA in the presence or absence of dexamethasone and forskolin (cAMP) after 2 days in culture. Exposure to dexamethasone, forskolin, and/or HGA was for 20 hr before harvesting. Five micrograms of total RNA was hybridized to the indicated probes.
NMO-1 expression in \( \text{c}^{14}\text{CoS}/\text{c}^{14}\text{CoS} \) hepatocytes is compatible with their inability to clear the tyrosine metabolites because of the lack of FAH. Hormone induction of NMO-1 in \( \text{c}^{14}\text{CoS}/\text{c}^{14}\text{CoS} \) hepatocytes might reflect activation of the tyrosine degradation pathway, because glucocorticoids and cAMP strongly induce TAT, the enzyme catalyzing the first reaction of the pathway. Therefore, these experiments imply that changes in levels of tyrosine metabolites can bring about secondary effects on gene expression characteristic of the \( \text{alf}/\text{hsdr-1} \) deletion phenotype that are not normally expressed in vitro.

**Discussion**

*Identification of the Fah gene as a candidate for \( \text{alf}/\text{hsdr-1} \)*

In this paper we show that two aspects of the \( \text{c}^{14}\text{CoS} \) phenotype, the reduced expression of hormone-inducible genes and induction of NMO-1 mRNA, depend on loss of a largely natural ~315 kb of the ~3800-kb \( \text{c}^{14}\text{CoS} \) deletion, the same region that is essential for neonatal survival (Russell et al. 1982; Schedl et al. 1992). In addition, both negative and positive effects on gene expression, as well as the ultrastructural lesions (Trigg and Gluecksohn-Waelsch 1973), are restricted to the same two cell types. Therefore, it is probable that these abnormalities result from the absence of a single gene, located partly or entirely in this ~315-kb region. Zoo blot and Northern analysis with probes from this region identified the gene for FAH. The Fah gene has been identified independently by Klebig et al. (1992).

Three lines of evidence strongly support Fah as a candidate gene for \( \text{alf}/\text{hsdr-1} \). First, there is a striking concordance between the cell types that normally express high-levels of FAH mRNA and those in which the phenotype develops. Second, inherited FAH deficiency in man leads to a fatal infant liver disease (Kvittingen 1986; Lindblad et al. 1977; Laberge et al. 1990), although inducers seem to be characterized by electrophilic olefin or acetylene bonds (Talalay et al. 1988). FAH possesses an olefinic bond flanked by electron-withdrawing keto and/or carboxyl groups (Fig. 3B), which suggests that it falls into this class of inducers. Consistent with a detoxifying role, NMO-1 mRNA is strongly elevated in \( \text{c}^{14}\text{CoS} \) homozygotes in the cell types that should normally contain the highest levels of FAH, those in which tyrosine degradates predominate, and is among the earliest components of the phenotype (this paper; Petersen et al. 1989). The possibility that abnormal concentrations of tyrosine metabolites can lead to NMO-1 induction is reinforced by the sensitivity of NMO-1 mRNA levels in cultured \( \text{c}^{14}\text{CoS}/\text{c}^{14}\text{CoS} \) hepatocytes toward HGA (HGA does not have the same electrophilic character as FAH). In this context, it is interesting that activities of GST B and UDP-glucuronosyltransferase—two other enzymes involved in detoxification and, like NMO-1, members of the \( \text{Ah} \) battery—were found to be increased in newborn \( \text{c}^{14}\text{CoS}/\text{c}^{14}\text{CoS} \) livers more than a decade ago (Thaler et al. 1976; Gatmaitan et al. 1977).

A second component of the early response to FAA may be the induction of gadd transcripts: gadd153 mRNA is present already at fetal day 16 in \( \text{c}^{14}\text{CoS}/\text{c}^{14}\text{CoS} \) liver (Fornace et al. 1989). The gadd genes were identified on the basis of induction by DNA damage (Fornace et al. 1988). The three gadd transcripts elevated in \( \text{c}^{14}\text{CoS}/\text{c}^{14}\text{CoS} \) belong to a class most sensitive to DNA damage by alkylating agents (Fornace et al. 1989), and might owe their induction to the presumed alkylating properties of FAA. Expression of the early response gene c-fos is also responsive to DNA damage by alkylating agents (Hollander and Fornace 1989), and the sustained, high-level expression of FOS mRNA in \( \text{c}^{14}\text{CoS}/\text{c}^{14}\text{CoS} \) liver might be a manifestation of this.

Overt signs of intracellular damage in \( \text{c}^{14}\text{CoS}/\text{c}^{14}\text{CoS} \) mice are the intracellular membrane abnormalities that become apparent at fetal day 18 and, like other aspects of the phenotype, are confined to liver and kidney (Trigg...
The failure of perinatal activation of hormone-dependent genes

The failure to activate hormone-dependent genes, such as TAT and PEPCk (Schmid et al. 1985; Loose et al. 1986), is the aspect of the alf/hsdr-1 deletion phenotype that has generated the most interest. Inducibility by glucocorticoids and/or the cAMP pathway was found to be a property common to mRNAs reduced in mutant liver (Ruppert et al. 1990). A selective effect on hormone-inducibility was suggested, moreover, by the impaired glucocorticoid, but normal heavy metal, response of the metallothionein gene Mt-1 in mutant livers (DeFranco et al. 1988). One observation that casts doubt on the notion that alf/hsdr-1 encodes a factor that participates directly in hormonal activation, however, and that is easier to reconcile with a secondary effect on gene expression, is our finding that the TAT and SAMS genes displayed equally strong hormone responses in c\(^{14C}S/c^{14C}S\) and wild-type hepatocytes cultured from fetal livers. The unimpaired hormone response of primary cultures of c\(^{14C}S/c^{14C}S\) hepatocytes suggests that tyrosine metabolites do not normally reach harmful levels in cultured hepatocytes, at least under our culture conditions.

Three mechanisms contributing to the failure to activate hormone-dependent genes can be envisaged considering that the signal transduction pathways for glucocorticoids and cAMP are apparently intact (Ruppert et al. 1990; DeFranco et al. 1991). The first might involve reduced expression of transcription factors: The mRNAs for hepatic nuclear factors HNF-1 and HNF-4 are affected most strongly (Gonzalez et al. 1990; Tönjes et al. 1992), while HNF-3 and C/EBP show modest reductions (Mc-Knight et al. 1989; Ruppert et al. 1990; Tönjes et al. 1992). One or more of these factors might be required for hormone-dependent and/or high-level expression of the TAT and PEPCk genes. The liver-specific enhancer 3.6 kb upstream of the rat TAT gene (Boshart et al. 1990) contains a cAMP-responsive element recognized by cAMP-responsive element binding (CREB) protein (Nichols et al. 1992) and a binding site for HNF-4 (D. Niewolik, pers. comm.). Both elements are required for enhancer function (Boshart et al. 1990). Similarly, the glucocorticoid-dependent enhancer at $-2.5$ kb of the rat TAT gene not only contains binding sites for the glucocorticoid receptor (GR) but also for other factors (Becker et al. 1986; Reik et al. 1991), which might include CCAAT box/enhancer-binding protein (C/EBP) (Grange et al. 1991) and HNF-3 (D. Nitsch, pers. comm.). These accessory factors are likely to be essential for full function of the $-2.5$-kb enhancer (Jantzen et al. 1987; D. Niewolik, pers. comm.).

A second mechanism is suggested by the recent discovery that the product of the gadd153 gene, which is elevated early in c\(^{14C}S/c^{14C}S\) liver (Fornace et al. 1989), is homologous to CHOP-10 (Ron and Habener 1992). CHOP-10 was identified by its ability to form heterodimers with the transcription factors C/EBP\(\alpha\) and C/EBP\(\beta\) via a leucine zipper. CHOP-10 prevents recognition of C/EBP-binding sites by C/EBP\(\alpha\) and C/EBP\(\beta\) and inhibits trans-activation of C/EBP-dependent promoters (Ron and Habener 1992).

Third, we detected a high and sustained level of c-fos transcripts in c\(^{14C}S/c^{14C}S\) liver before and after birth, which might be related to alkylation damage induced by FAA. Transfection studies have suggested that FOS protein can antagonize glucocorticoid induction (Jonat et al. 1990; Lucibello et al. 1990; Yang-Yen et al. 1990; Shemeshedini et al. 1991), although the mechanism is unclear.

In rat hepatoma cells treated with the phorbol ester TPA, induction of FOS mRNA is associated with decreased glucocorticoid-induced levels of TAT mRNA (A. Reik, pers. comm.).

The alf/hsdr-1-deficient mouse as model for the human genetic disease tyrosinemia type I

The greatest hint that absence of FAH could be responsible for the alf/hsdr-1 deletion phenotype came from comparison with the human deficiency state. FAH deficiency is the primary defect in the autosomal recessive disease hereditary tyrosinemia type I (HT), a well-characterized disorder of tyrosine metabolism (Kvittingen 1986; Goldsmith and Laberge 1989). In its more common, acute form, HT presents as an infantile liver failure, and death normally ensues within a few months of birth unless the liver is transplanted. More chronic liver failure is associated with residual enzyme activity and protein (Tanguay et al. 1990) and is characterized by a high incidence of hepatocellular carcinoma in the first decade of life (Weinberg et al. 1976; Russo and O'Regan 1990). In contrast to the relatively more protracted human disease, the alf/hsdr-1 phenotype is highly uniform...
and lethality occurs a few days after the first manifestations of FAH deficiency. Because abnormalities in FAH deficiency arise from the presence of harmful tyrosine metabolites, the phenotype depends, in part, on the ability to detoxify these compounds. In addition, the human and mouse phenotypes that can be compared are diverse secondary effects arising from the same primary defect. The example of hypoxanthine phosphoribosyltransferase deficiency in man (Lesch-Nyhan syndrome) and mouse underscores the importance of secondary metabolic differences in determining very different outcomes (Hooper et al. 1987; Kuehn et al. 1987). Nevertheless, the comparison with HT supports our conclusion that FAH deficiency is the basis for neonatal lethality in c14C°S/c14C°S mice. Abnormalities are most pronounced in the two organs that normally express the highest levels of FAH and are active in tyrosine degradation. The limitation of ultrastructural and histological changes to these organs is consistent with a very local site of action of the damaging intermediates (Trigg and Gluecksohn-Waelsch 1973; Dehner et al. 1989; Russo and O'Regan 1990). Some of the enzymes in which expression is strongly reduced in c14C°S/c14C°S mice have been assayed in HT material. Decreases in TAT activity have been described (Gaull et al. 1970; Furukawa et al. 1984; Stoner et al. 1984; Laberge et al. 1985), as well as reductions in SAMS and other enzymes of methionine metabolism (Gaull et al. 1970). It will be of great interest to investigate whether other changes characteristic of the alf/hsdr-1 deletion phenotype are represented in HT. We envisage that the c14C°S/c14C°S mouse will provide a useful model that will deepen the understanding of FAH deficiency in man.

Materials and methods

Construction of cDNA libraries, isolation and characterization of FAH cDNA clones

Poly(A)+ RNA from livers of newborn c14C°S/c14C°S mice was used for construction of a directional cDNA library in hZAPII (Stratagene), essentially as described previously (Ruppert et al. 1988; Schöler et al. 1990). Of the original 5 x 107 recombinants, 3 x 104 PFU were amplified on Escherichia coli PLK-F and screened with probes from genomic clones RN.Fa and RN.Fd (Sched et al. 1992). Twelve independent cDNA clones were isolated and characterized by restriction enzyme analysis and DNA sequencing from their 5' and 3' ends (Lucow and Schütz 1991). Sequencing of the coding region of four cDNAs, including pmcFAH7 and pmcFAH3, was completed with internal oligonucleotide primers. A detailed description of the FAH cDNA and genomic clones will be presented elsewhere (G. Kelsey, unpubl.).

RNA preparation and Northern blot analysis

Fetal liver and kidney RNAs were prepared from albino c14C°S/c14C°S and wild-type mice from organs pooled from each stage, and tissue RNAs were prepared from wild-type adult mice and rats as described previously (Ruppert et al. 1990). Liver RNAs for time points after birth have been described (Ruppert et al. 1990). Northern blot analysis with antisense riboprobes was performed as described previously (Ruppert et al. 1990). Southern blot analysis

Southern blot analysis of albino deletion DNAs was performed as described previously (Kelsey et al. 1992). Hybridization was with a gel-purified insert from pmcFAH3 labeled by random priming (Feinberg and Vogelstein 1984).

In situ hybridization

Livers and kidneys of day 18 lethal albino c14C°S/c14C°S and normal littermates c14C°S/c14C°S were fixed in 4% paraformaldehyde and embedded in paraffin according to Duboule and Dollé (1989). Hybridizations were performed on 5- to 10-μm sections with sense and/or antisense RNA probes for FAH, PEPCK, and NMO-1 labeled with [α-35S]UTP (Duboule and Dollé 1989). Sections were exposed for 4–19 days, developed with Kodak D19, and counterstained with hematoxylin and eosin.

Probes used in this analysis

Probes for TAT, PEPCK, and X1 have been described (Ruppert et al. 1990). cDNAs X2 and X5 (Ruppert et al. 1990) have been identified to correspond to SAMS and CoAS, respectively, by recent sequence comparisons (Horikawa et al. 1989; Ayte et al. 1990, details available on request). The FAH probe was the near full-length cDNA clone pmcFAH3, which contains a deletion of untranslated sequences at the 3' end of the cDNA. A probe for NMO-1 was made by polymerase chain reaction (PCR) with oligonucleotides corresponding to position 41–5'-CTTGA-CACTAGTGATCCGCCCCAACTTCTGG-3'—and position 885–5'-CTAGCTTAGATCTGGTTGTCGGCTGGAATCC-3'—of the rat NMOR1 cDNA (Robertson et al. 1986) and containing engineered BamHI and BgIII restriction sites. Reverse transcription/PCR was performed on RNA from the rat hepatoma cell line FTO-2B, under conditions described by Ruppert et al. (1992), and an 850-bp BamHI–BgIII fragment was subcloned into Bluescribe [Stratagene] to yield plasmid pNC-MOR. Identity to the published sequence was confirmed by partial sequence analysis.

Isolation and culture of fetal hepatocytes

Primary hepatocyte cultures were prepared as described by Yeoh et al. (1979) with slight modifications. Hepatocytes enriched from livers of day 17.5 albino c14C°S/c14C°S and normal littermates c14C°S/c14C°S or c14C°S/c14C°S were plated at a density of approximately two cells per 5-cm collagen-coated culture dish in Dulbecco's modified Eagle medium (DMEM/HamF12 medium containing 10% fetal calf serum, insulin (1 μg/ml), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml). Cells were cultured for 2–3 days before induction with hormones in media lacking insulin. Glucocorticoid induction was for the final 17 hr (1 μM dexamethasone) and cAMP induction (0.5 mM 3',5'-chlorophenylthio-cAMP; CPT-cAMP, Boehringer Mannheim) was for the final 5 hr before harvesting. Two conditions of HGA treatment were used. After 2 days of culture, hepatocytes were induced with 1 μM dexamethasone and 10 μM forskolin, 500 μM HGA (Sigma) was added after 6 hr, and cells were cultured 17 hr before harvesting. Alternatively, HGA (100 or 500 μM) was added at the same time as dexamethasone (1 μM) and forskolin (10 μM), and cells were harvested 20 hr later. RNAs were prepared according to Chomczynski and Sacchi [1987].
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Note added in proof

The FAH amino acid sequence has been deposited in the EMBL/GenBank data libraries.

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