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 hydrolase (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 hydrolase; 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 *hsdr-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-

¹Present address: Howard Hughes Medical Institute, Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, California 94720 USA.

²These authors contributed equally to this work.

³Corresponding author.

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-glucuronyltransferase 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-1n*, 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^{14CoS}* [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^{10R75M}* and *c^{11DSD}*, 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 hydrolase (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^{14CoS}*, 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

Figure 1. The *alf/hsdr-1* deletion phenotype develops before birth in liver and kidney. Total RNA (10 μ g) from livers of lethal albino mice and wild-type littermates at time points before birth (16.5, 17.5, and 18.5 days of gestation) and after birth (0–1 to 48 hr) were analyzed by Northern blot hybridization with the following cDNA probes: TAT, PEPCK, CoAS, SAMS, NMO-1, and FOS (*c-fos*). X1 is an unidentified cDNA isolated by differential screening (Ruppert et al. 1990). The cDNAs for CoAS and SAMS correspond, respectively, to cDNAs X5 and X2 isolated by differential screening (Ruppert et al. 1990). (Inset below) A similar analysis for NMO-1 mRNA in kidney (5 μ g of total RNA) from lethal albino mice [(A) *c^{14CoS}/c^{14CoS}*] and normal littermates [(W) wild type]. (Newb.) Newborn mice 1–4 hr after birth. Each RNA was prepared from organs pooled for each time point.

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 c^{14CoS}/c^{14CoS} liver (Tönjes et al. 1992).

The mRNA for the detoxifying enzyme NMO-1 is highly induced in c^{14CoS}/c^{14CoS} 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 c^{14CoS}/c^{14CoS} 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 c^{14CoS}/c^{14CoS} liver differed strikingly in two respects: FOS mRNA was clearly elevated at fetal day 18.5, and a high level was maintained for several hours after birth (Fig. 1).

The *alf/hsdr-1* deletion phenotype results from loss of a gene(s) located in a ~315-kb interval

Much of the analysis of the *alf/hsdr-1* phenotype has concerned deletion c^{14CoS} because, apart from removal of the *c* locus, *alf/hsdr-1* is the only function affected. Although it was likely from complementation analysis (Gluecksohn-Waelsch 1979; Russell et al. 1982) that *alf/hsdr-1* is defined by the proximal region of the c^{14CoS} deletion, the possibility remained that separate loci were responsible for each aspect of the c^{14CoS} deletion phenotype, particularly in light of the finding that the c^{14CoS} deletion is ~3800 kb (Kelsey et al. 1992). The compound heterozygote c^{11DSD}/c^{14CoS} is complemented for neonatal lethality and glucose-6-phosphatase deficiency (Russell et al. 1982), and physical mapping indicates that only the most proximal ~315 kb of the c^{14CoS} deletion is restored in the compound heterozygote [(Schedl et al. 1992) shown schematically in Figure 2A]. To examine whether this region was essential for the correct expression of our set of affected genes, the abundance of their mRNAs was compared in newborn livers from c^{14CoS}/c^{14CoS} and c^{11DSD}/c^{14CoS} mice. Whereas TAT, PEPCK, SAMS, and CoAS mRNAs are severely reduced in c^{14CoS} homozygotes, they are present in normal amounts in the c^{11DSD}/c^{14CoS} heterozygote (Fig. 2B). In addition, the elevation of NMO-1 mRNA was not seen in c^{11DSD}/c^{14CoS} liver. Therefore, all facets of the c^{14CoS} 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 c^{14CoS} and c^{11DSD} deletions.

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

Nearly one-third of the region between the proximal

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; (○) the centromere. Below, the proximal extensions of the c^{14CoS} , c^{10R75M} , and c^{11DSD} 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 c^{11DSD} and c^{14CoS} deletion breakpoints; the broken arrow indicates the possibility that a candidate gene might extend beyond the proximal extremes of the c^{10R75M} or c^{14CoS} deletions. The proximal breakpoints of the c^{10R75M} and c^{14CoS} 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 μ g) isolated shortly after birth from the livers of lethal albino mice (c^{14CoS}/c^{14CoS}), and their normal littermates (c^{ch}/c^{ch} or c^{ch}/c^{14CoS}), or complemented double heterozygotes (c^{11DSD}/c^{14CoS}) and their normal littermates (c^{11DSD}/c^{ch} or c^{14CoS}/c^{ch}) were analyzed by Northern blot hybridization with the indicated cDNA probes (for abbreviations, see Fig. 1).

boundaries of the c^{14CoS} and c^{11DSD} 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^{14CoS} 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^{14CoS} 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^{14CoS}/c^{14CoS} 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/hedr-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^{14CoS} 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/hedr-1*, we investigated how the *Fah* gene was altered by the deletions. Homozygous c^{10R75M} and c^{14CoS} DNAs were compared with wild-type control c^{ch}/c^{ch} and C3H DNAs on Southern blots hybridized with a near full-length *Fah* cDNA, pmcFAH3 (Fig. 4A). Altered restriction fragments were detected in both deletion DNAs, from which it was concluded that the c^{14CoS} 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^{14CoS} homozygotes

Figure 3. A gene identified within the *alf/hedr-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 c^{14CoS} and c^{10R75M} deletion breakpoints within the mouse *Fah* gene. (A) Southern blots of *Eco*RI, *Hind*III, *Xba*I and *Bam*HI-digested DNAs from mice homozygous for the c^{14CoS} or c^{10R75M} deletions and control homozygous c^{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 *Eco*RI and *Hind*III fragments, which comigrate with other exon fragments (G. Kelsey, unpubl.). The numbers adjacent to the c^{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 *Eco*RI site absent on the c^{ch} allele (identified by an asterisk in B) gives rise to a larger fragment for exons 11–13 [shown as (11–13)]. (○) An *Eco*RI fragment in the c^{14CoS} lane suspected to be a partial digestion product. (B) The extents of the c^{14CoS} and c^{10R75M} deletions are shown as solid arrows below the map of *Eco*RI, *Hind*III, *Xba*I, and *Bam*HI restriction sites of the mouse *Fah* gene. The c^{10R75M} deletion breakpoint has been mapped between *Bam*HI and *Hind*III 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 *Eco*RI site absent on the c^{ch} allele is marked by an asterisk (*).

(Fig. 5B) nor c^{10R75M} homozygotes (Klebig et al. 1992). The failure of the *Fah* gene to be expressed from the c^{10R75M} 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

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^{14CoS} 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^{14CoS}/c^{14CoS} liver, FAH mRNA expression was similar to wild type in newborn liver of the complemented heterozygote c^{11DSD}/c^{14CoS} (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^{14CoS}/c^{14CoS} 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^{14CoS}/c^{14CoS} kidney (Fig. 6E). NMO-1 expression was detected only in c^{14CoS}/c^{14CoS} 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 expression was confined to hepatocytes, and NMO-1 expression was restricted to c^{14CoS}/c^{14CoS} 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/hedr-1* deletion phenotype.

Expression of the *alf/hedr-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^{14CoS} deletion raised the possibility that the alterations in gene expression characteristic of the *alf/hedr-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/hedr-1*. Livers were removed from day 17.5 or 18.5 c^{14CoS}/c^{14CoS} (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^{14CoS}/c^{14CoS} hepatocyte cultures, TAT mRNA accumu-

Figure 6. (See facing page for legend.)

Figure 7. Expression of the *alf/hsdr-1* deletion phenotype in primary hepatocytes. (A) Primary hepatocytes from wild-type and lethal albino (c^{14CoS}/c^{14CoS}) 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 μ g of total RNA, with the cDNA probes indicated. Hybridization with a transferrin cDNA probe is shown (Tf). (B) Primary hepatocytes from albino lethal (c^{14CoS}/c^{14CoS}) and wild-type mice were treated with HGA (500 μ M) 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 (c^{14CoS}/c^{14CoS}) and wild-type mice were treated with 100 or 500 μ M 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.

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 FAH mRNA, which appeared only in cultures of wild-type hepatocytes, and was found to be induced by dexamethasone (Fig. 7A). The apparently normal expression of TAT and SAMS mRNAs in isolated c^{14CoS}/c^{14CoS} hepatocytes is in striking contrast to the phenotype in newborn c^{14CoS}/c^{14CoS} liver and argues against the notion that *alf/hsdr-1* encodes a factor that participates directly in the hormone activation or high-level expression of these genes.

To test the effect of an increased intracellular concentration of FAA and/or MAA on gene expression, hepatocyte cultures were exposed to the tyrosine metabolite homogentisate [(HGA) see Fig. 3B]. The use of oral loading of HGA to expose the carrier state in inherited human FAH deficiency (Laberge et al. 1990) was taken as an indication that HGA would be metabolized in hepatocytes and that normal levels of FAH activity could be saturated. In addition, the experience of alkaptonuria, an inborn error of metabolism in which HGA metabolism is

blocked (La Du 1989), was taken to indicate that HGA would not itself be toxic to hepatocytes.

HGA treatment (500 μ M) was found to reduce TAT and SAMS mRNAs in cultures induced with dexamethasone and forskolin close to uninduced levels, both in wild-type and c^{14CoS}/c^{14CoS} 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 c^{14CoS}/c^{14CoS} hepatocytes displaying an apparently greater increase (Fig. 7B). A differential response in NMO-1 expression between wild-type and c^{14CoS}/c^{14CoS} 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 c^{14CoS}/c^{14CoS} hepatocytes treated with 500 μ M 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 c^{14CoS}/c^{14CoS} hepatocytes even in the absence of HGA. A stronger effect on

Figure 6. In situ hybridization identifies the major site of *Fah* gene expression in kidney. Paraffin sections of fetal day 18 wild-type kidney were hybridized with antisense probes for PEPCK (A,B), FAH (C,D), and NMO-1 (F). Sections from fetal day 18 c^{14CoS} homozygous kidney were hybridized with antisense FAH (E) and NMO-1 (G,H) probes. (A,C,E,F,G) Dark-field illumination; (B,D,H) bright-field illumination to reveal predominant localization of grains on tubule cells and absence of grains from glomeruli.

NMO-1 expression in c^{14CoS}/c^{14CoS} hepatocytes is compatible with their inability to clear the tyrosine metabolites because of the lack of FAH. Hormone induction of NMO-1 in c^{14CoS}/c^{14CoS} 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 *alf/hsdr-1* deletion phenotype that are not normally expressed in vitro.

Discussion

Identification of the Fah gene as a candidate for alf/hsdr-1

In this paper we show that two aspects of the c^{14CoS} phenotype, the reduced expression of hormone-inducible genes and induction of NMO-1 mRNA, depend on loss of the most proximal ~315 kb of the ~3800-kb c^{14CoS} 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 *alf/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), which has suggestive parallels with the *alf/hsdr-1* deletion phenotype. Third, based on the knowledge that FAH is an enzyme of tyrosine metabolism, we induced changes in gene expression in cultured hepatocytes similar to those seen in the mutant liver by treatment with a tyrosine metabolite. Recently, we have been able to show that an FAH cDNA transgene can restore viability to c^{14CoS} homozygotes (G. Kelsey, S. Ruppert, and F. Beermann, unpubl.).

Accumulation of reactive tyrosine metabolites could cause the alf/hsdr-1 deletion phenotype

The c^{14CoS} phenotype was interpreted as indicating that the deletion removes a locus, *alf* or *hsdr-1*, required for perinatal activation of hormone-dependent genes in liver (Gluecksohn-Waelsch 1987; McKnight et al. 1989; Ruppert et al. 1990). In addition, a locus encoding a repressor of the *Nmo-1* and *gadd* genes, designated *Nmo-1n*, has been proposed to map within the deletion (Petersen et al. 1989; Nebert et al. 1990). It might seem surprising that we should advance a gene for a metabolic enzyme, FAH,

as a candidate for both *alf/hsdr-1* and *Nmo-1n*, but we can now offer an alternative explanation for the c^{14CoS} deletion phenotype.

FAH deficiency blocks the tyrosine degradation pathway beyond the hydrolysis of FAA. FAA is strongly electrophilic, adducts rapidly with glutathione (Edwards and Knox 1956; Laberge et al. 1990), and could react with thiol or other nucleophilic groups on macromolecules. Although toxicity of FAA has not been shown directly, it is suspected to be an alkylating agent and is presumed to initiate the cellular damage accompanying FAH deficiency in man (Lindblad et al. 1977; Kvittingen 1986; Laberge et al. 1986). The consequences of an accumulation of FAA in perinatal mouse liver might be expected to include early responses, which attempt to detoxify the liver and repair damage caused, and secondary effects, such as those on gene expression.

An early detoxification response could be the elevated expression of NMO-1. NMO-1 is induced by and metabolizes a wide variety of xenobiotics (Talalay and Prochaska 1987; Lind et al. 1990), although inducers seem to be characterized by electrophilic olefin or acetylene bonds (Talalay et al. 1988). FAA 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 c^{14CoS} homozygotes in the cell types that should normally contain the highest levels of FAH, those in which tyrosine degradation predominates, 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 c^{14CoS}/c^{14CoS} hepatocytes toward HGA (HGA does not have the same electrophilic character as FAA). 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 (*Ah*) battery—were found to be increased in newborn c^{14CoS}/c^{14CoS} 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 c^{14CoS}/c^{14CoS} 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 c^{14CoS}/c^{14CoS} 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 c^{14CoS}/c^{14CoS} liver might be a manifestation of this.

Overt signs of intracellular damage in c^{14CoS}/c^{14CoS} 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

and Gluecksohn-Waelsch 1973). These lesions might arise from alkylation of membrane components or oxidative stress.

The final component of the c^{14CoS} phenotype is the reduced expression of hormone-dependent genes that becomes most pronounced around birth, a timing consistent with an effect secondary to cellular responses or damage elicited by FAA. The sequence of events leading to reduced expression of any one of the affected genes may be complex and brought about by a combination of factors (as discussed below).

It might seem paradoxical that consequences of FAH deficiency should occur in fetal liver and kidney, while high-level expression of TAT, the enzyme catalyzing the first step in tyrosine degradation, develops exclusively in the liver around birth and is severely reduced in c^{14CoS} homozygotes. The explanation for this paradox seems to be that tyrosine is also deaminated by aspartate aminotransferase, which is active in several mammalian tissues, including fetal liver and kidney (Fellman et al. 1972a; Yeoh et al. 1979; Hargrove and Mackin 1984), and is not reduced in c^{14CoS}/c^{14CoS} liver (Thorndike et al. 1973). The phenotype is restricted to the liver and kidney because generation of the most damaging tyrosine metabolites is limited by the tissue specificity of subsequent enzymes in the pathway (Fellman et al. 1972b).

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^{14CoS}/c^{14CoS} and wild-type hepatocytes cultured from fetal livers. The unimpaired hormone response of primary cultures of c^{14CoS}/c^{14CoS} 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. Niewollik, 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. Niewollik, pers. comm.).

A second mechanism is suggested by the recent discovery that the product of the *gadd153* gene, which is elevated early in c^{14CoS}/c^{14CoS} 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 α and C/EBP β via a leucine zipper. CHOP-10 prevents recognition of C/EBP-binding sites by C/EBP α and C/EBP β 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^{14CoS}/c^{14CoS} 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; Shemshedini 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 c^{14CoS}/c^{14CoS} 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 c^{14CoS}/c^{14CoS} 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/hedr-1* deletion phenotype are represented in HT. We envisage that the c^{14CoS}/c^{14CoS} 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 c^{ch}/c^{ch} mice was used for construction of a directional cDNA library in λ ZAPII (Stratagene), essentially as described previously (Ruppert et al. 1988; Schöler et al. 1990). Of the original 5×10^7 recombinants, 3×10^5 PFU were amplified on *Escherichia coli* PLK-F' and screened with probes from genomic clones RN.Fa and RN.Fd (Schedl et al. 1992). Twelve independent cDNA clones were isolated and characterized by restriction enzyme analysis and DNA sequencing from their 5' and 3' ends (Luckow 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 (c^{14CoS}/c^{14CoS}) 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 (c^{14CoS}/c^{14CoS}) and normal littermates (c^{ch}/c^{ch} or c^{ch}/c^{14CoS}) were fixed in 4% paraformaldehyde and embedded in paraffin according to Duboule and Dollé (1989). Hybridizations were performed on 5- to 10- μ m 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; Ayté 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'-CTTGACACTAGGATCCGCCCAACTTCTGG-3'—and position 885–5'-CTAGCTTAGATCTGGTTGTCGGCTGGAATCC-3'—of the rat NMOR₁ cDNA (Robertson et al. 1986) and containing engineered *Bam*HI and *Bgl*II 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 *Bam*HI–*Bgl*II fragment was subcloned into Bluescribe (Stratagene) to yield plasmid prcNMOR. 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 (c^{14CoS}/c^{14CoS}) and normal littermates (c^{ch}/c^{ch} or c^{ch}/c^{14CoS}) were plated at a density of approximately two livers per 5-cm collagen-coated culture dish in Dulbecco's modified Eagle medium (DMEM)/HamF12 medium containing 10% fetal calf serum, insulin (1 μ g/ml), penicillin (100 U/ml), and streptomycin sulfate (100 μ g/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 μ M dexamethasone) and cAMP induction (0.5 mM *p*-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 μ M dexamethasone and 10 μ M forskolin, 500 μ M HGA (Sigma) was added after 6 hr, and cells were cultured 17 hr before harvesting. Alternatively, HGA (100 or 500 μ M) was added at the same time as dexamethasone (1 μ M) and forskolin (10 μ M), 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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