

Disruption of Cholesterol 7 α -Hydroxylase Gene in Mice

I. POSTNATAL LETHALITY REVERSED BY BILE ACID AND VITAMIN SUPPLEMENTATION*

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Mice deficient in cholesterol 7 α -hydroxylase, the rate-limiting enzyme of bile acid biosynthesis, were constructed by targeted disruption of the *Cyp7* gene. The introduced mutation removed exons 3–5 of the gene and gave rise to a null allele that encoded no immunoreactive or enzymatically active protein. Heterozygous carriers of the disrupted gene (*Cyp7*^{+/-}) were phenotypically normal. Homozygous animals (*Cyp7*^{-/-}) appeared normal at birth, but died within the first 18 days of life. Approximately 40% of the animals died between postnatal days 1 and 4 and 45% between days 11 and 18. The addition of vitamins to the water of nursing mothers prevented deaths in the early period, whereas the addition of cholic acid to chow prevented deaths in the later period. Newborn *Cyp7*^{-/-} mice whose mothers were maintained on unsupplemented chow failed to gain weight at a normal rate and developed oily coats, hyperkeratosis, and apparent vision defects. These symptoms waned at 3 weeks of life, and their disappearance was accompanied by a marked increase in survival. In the accompanying study, the induction of an alternate pathway of bile acid biosynthesis is shown to underlie this unusual time course (Schwarz, M., Lund, E. G., Setchell, K. D. R., Kayden, H. J., Zerwekh, J. E., Björkhem, I., Herz, J., and Russell, D. W. (1996) *J. Biol. Chem.* 271, 18024–18031). We conclude that cholesterol 7 α -hydroxylase is an essential enzyme for normal postnatal development.

Cholesterol (cholest-5-en-3 β -ol) is metabolized in mammals into two essential classes of compounds, bile acids and steroid hormones. Approximately 400 mg of cholesterol is converted on a daily basis into bile acids by the human liver, while endocrine glands convert another 50 mg into steroid hormones (1). Thus, the bile acid pathway accounts for the majority of cholesterol catabolism, and it plays a crucial role in cholesterol homeostasis.

Bile acid synthesis involves the addition of hydroxyl groups to the ring structure of cholesterol and the oxidation and shortening of the side chain (2). These reactions, which are carried out by more than a dozen hepatic enzymes, produce the primary bile acids cholic acid and chenodeoxycholic acid. Following synthesis, bile acids are secreted from the hepatocyte into

bile as glycine or taurine conjugates and transported to the lumen of the small intestine. In the intestine, bile acids act as detergents to solubilize ingested fats, sterols, and fat-soluble vitamins and aid the uptake of these essential nutrients by cells lining the proximal segment of the small intestine. Bile acids are reclaimed in the distal segment of the intestine by a specific bile acid transporter (3) and then transported back to the liver in the portal circulation. A second bile acid transporter in the sinusoidal membrane of hepatocytes clears bile acids from the blood (4).

The importance of bile acids in fat metabolism and nutrition is underscored by the abnormal phenotypes associated with naturally occurring mutations that disrupt their biosynthesis. Mutations in genes encoding enzymes that modify either the ring structure (5, 6) or the side chain (7) of cholesterol cause a spectrum of phenotypes, including improper liver function (cholestasis), fat malabsorption (steatorrhea), premature atherosclerosis, and central nervous system dysfunction (8). These experiments of nature indicate that defects in the biosynthetic pathway of bile acids have important phenotypic consequences for both cholesterol and lipid metabolism.

No mutations have yet been found in cholesterol 7 α -hydroxylase (hereafter referred to as 7 α -hydroxylase; cholesterol 7 α -monooxygenase, EC 1.14.13.17), a microsomal cytochrome P450 enzyme that catalyzes the first step in the microsomal pathway of bile acid biosynthesis (2, 9). This enzyme catalyzes the 7 α -hydroxylation of cholesterol to produce 7 α -hydroxycholesterol (cholest-5-ene-3 β ,7 α -diol), which is subsequently converted into cholic acid and chenodeoxycholic acid. The expression of the 7 α -hydroxylase gene (*CYP7* in humans and *Cyp7* in mice) is enhanced when bile acids are depleted from the enterohepatic circulation and is suppressed when excess bile acids are present in the diet (2).

To gain further insight into the physiological role of 7 α -hydroxylase and the importance of the microsomal pathway of bile acid biosynthesis initiated by this enzyme, we used homologous recombination in embryonic stem cells to produce a strain of mice that transmit a null mutation in the *Cyp7* gene. The analysis of homozygous animals reveals a complex phenotype consistent with a crucial role for this enzyme and for bile acid biosynthesis in early postnatal life.

EXPERIMENTAL PROCEDURES

Construction of Targeting Vector—A 12-kilobase (kb)¹ fragment of the *Mus musculus* 7 α -hydroxylase gene was enriched by sucrose gradient density ultracentrifugation of *Bam*HI-digested genomic DNA isolated from the 129Sv strain. The resulting fraction was ligated into the bacteriophage λ DashII vector (Stratagene), and recombinant clones harboring the desired insert were identified by screening with a cDNA probe derived from the coding region of the mouse 7 α -hydroxylase mRNA. The cDNA probe was originally amplified from mouse liver mRNA using oligonucleotide primers derived from the rat cDNA sequence (10). A replacement-type targeting vector was constructed as

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¹ The abbreviations used are: kb, kilobase(s); ES, embryonic stem.

shown in Fig. 1A using the cloned genomic DNA fragment and a plasmid vector as templates. The short arm of the targeting vector (~1.2 kb) represented a *Bam*HI DNA fragment spanning intron 2 of the gene and was amplified from genomic DNA using two oligonucleotide primers (5'-primer = 5'-ATTTCATTACACTACTTCTGCGAAG-3' and 3'-primer = 5'-TGGGTCTATGCTTCTGTGTCCAAAT-3'). The long arm of the targeting vector (~12 kb) extended from a *Bam*HI site in intron 5 to a *Bam*HI site located 3' of the 7 α -hydroxylase gene. The *Pol2sneobpA* expression cassette (11) was positioned between the two murine DNA fragments (arms), and two copies of the herpes simplex virus thymidine kinase gene were located 5' of the short arm of the vector (see Fig. 1A).

Embryonic Stem Cell Culture—Mouse AB-1 embryonic stem (ES) cells derived from the 129Sv strain were cultured on leukemia inhibitory factor-producing STO feeder cells as described (12). Approximately 2×10^7 cells were electroporated (275 V, 330 millifarads) with 25 μ g/ml targeting vector linearized with *Sal*I using a device from Life Technologies, Inc. Electroporated cells were seeded onto irradiated (10,000 rads) feeder layers. After selection with 190 μ g/ml G418 and 0.25 mM 1-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)-5-iodouracil (Bristol Myers-Squibb), recombinant clones were identified by the polymerase chain reaction using a 5'-primer (5'-GAACCTCCTTTGGACAACGGGTTGA-3') located in exon 2 of the 7 α -hydroxylase gene and a 3'-primer (5'-AGGATTGGGAAGACAATAGCAGGCAT-3') located within the bovine growth hormone polyadenylation sequence of the *neo* expression cassette. Two independent stem cell clones (clones 86 and 193) containing a disrupted 7 α -hydroxylase allele were identified among 500 colonies screened. The homologous recombination events in these two clones were verified by Southern blot analysis of *Bgl*II-digested cellular DNA using an intron 1 hybridization probe (see Fig. 1B). Both ES cell clones were injected into C57Bl/6J blastocysts, yielding 18 chimeric males whose coat color (*agouti*) indicated a contribution from the stem cells ranging from 20 to 95%. Of these chimeric males, two (both derived from ES clone 193) transmitted the disrupted 7 α -hydroxylase gene through the germ line. All experiments reported here were performed with mixed strain (C57Bl/6J-129Sv) descendants (F2 and subsequent generations) of these animals.

7 α -Hydroxylase Assays—Enzyme assays, immunoblotting, and RNA blotting were carried out as described previously (10).

Animal Diets—The normal diet was mouse/rat diet 7001 (Harlan Teklad, Madison, WI) and contained $\geq 4\%$ (w/w) fat, $\geq 24\%$ (w/w) protein, and $\leq 5\%$ (w/w) fiber. Where indicated, this diet was supplemented with 1% (w/w) cholic acid (Sigma). A vitamin supplement (CrittterVites, Mardel Labs, Glendale Heights, IL) containing both water-soluble vitamins (thiamine, 180 mg/kg; riboflavin, 300 mg/kg; pantothenic acid, 600 mg/kg; niacin, 1500 mg/kg; vitamin B₁₂, 150 μ g/kg; vitamin B₆, 150 mg/kg; folic acid, 100 mg/kg; and ascorbic acid, 9000 mg/kg) and fat-soluble vitamins (vitamin A, 300,000 IU/kg; vitamin D₃, 50,000 IU/kg; vitamin E, 750 IU/kg; and menadione, 250 mg/kg) was added to water bottles at the concentration (1 g/liter) recommended by the manufacturer. Vitamin supplements were replaced on a daily basis.

Activity Test—The behavior of wild-type and 7 α -hydroxylase-deficient mice in an open field was determined using a modification of a test described by Aiba *et al.* (13).

RESULTS

Disruption of the murine 7 α -hydroxylase gene was carried out by standard methods in the 129Sv-derived ES cell line AB-1. A targeting vector was constructed as shown in Fig. 1A. Homologous recombination between this vector and a normal *Cyp7* allele (14) is expected to result in deletion of most of exon 3 and all of exons 4 and 5. Electroporation of the vector DNA followed by selection with neomycin and 1-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)-5-iodouracil yielded two ES cell lines that harbored the desired recombination event. For unknown reasons, the frequency of such clones was very low (0.4%). Both ES cell lines were injected into blastocysts derived from C57Bl/6J female mice and subsequently implanted in pseudopregnant females. Two of the resulting chimeric male animals transmitted the disrupted 7 α -hydroxylase gene through the germ line as judged by Southern blot analysis of genomic DNA derived from tail segments. The data of Fig. 1B show that crossing male and female animals heterozygous for the mutant allele resulted in offspring of both sexes with all three of the expected genotypes at the *Cyp7* locus. The 6.0-kb *Bgl*II DNA fragment character-

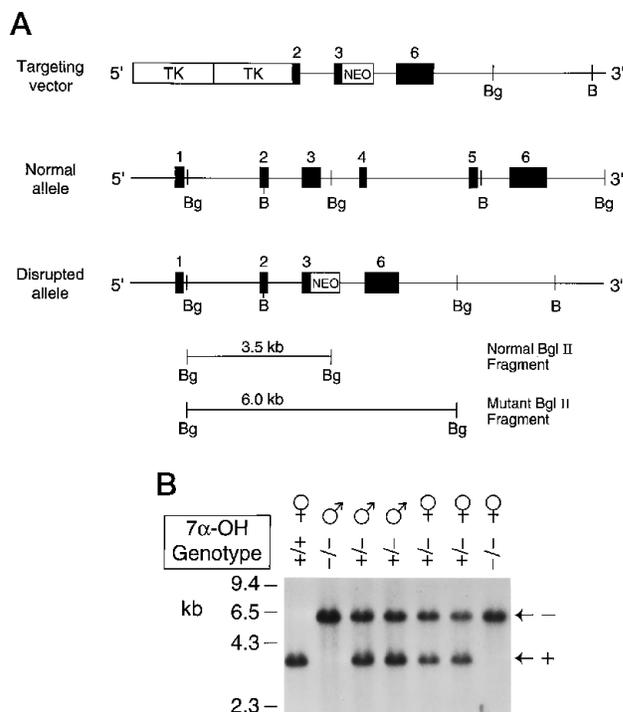


FIG. 1. Disruption of 7 α -hydroxylase gene in mice. A, schematics of the targeting vector, the normal 7 α -hydroxylase gene, and the predicted disrupted gene. Exons are numbered and indicated by black boxes. Introns and flanking regions of the gene are indicated by thin lines. TK, viral thymidine kinase genes; NEO, neomycin resistance gene; Bg, *Bgl*II restriction enzyme sites; B, *Bam*HI cleavage sites. The schemata are not drawn to scale. B, Southern blot analysis of genomic DNA isolated from the offspring of a cross between mice heterozygous for the 7 α -hydroxylase gene disruption. The genomic DNAs were digested with *Bgl*II, transferred to nylon membranes, and hybridized with a probe corresponding to intron 1 of the gene. This probe detects a 6.0-kb DNA fragment in the normal gene (+) and a 3.5-kb fragment in the disrupted gene (-).

istic of the disrupted gene and/or the 3.5-kb *Bgl*II DNA fragment characteristic of the normal gene was present in the expected Mendelian ratios in the offspring of this and other crosses.

To confirm that deletion of exons 3–5 of the gene abolished 7 α -hydroxylase enzyme activity, extracts of microsomal membranes prepared from the livers of animals with different genotypes were incubated with [¹⁴C]cholesterol in the presence of NADPH. As shown in Fig. 2A, wild-type mice express an enzyme activity capable of hydroxylating cholesterol at the 7 α -position (lanes 5 and 6). Extracts prepared from animals heterozygous for the disrupted *Cyp7* gene contain approximately one-half the amount of enzyme activity present in wild-type tissue (lanes 3 and 4), whereas extracts from *Cyp7*^{-/-} animals contain little or no cholesterol 7 α -hydroxylase activity (lanes 1 and 2). In control experiments, the production of 7 α -hydroxycholesterol in the assay was dependent on the presence of both NADPH cofactor and microsomal protein (lanes 7 and 8).

Immunoblotting of liver extracts prepared from mice homozygous for the disrupted gene also revealed the loss of a protein with a size and migration on SDS-polyacrylamide gels characteristic of 7 α -hydroxylase (Fig. 2B). An antibody raised against a highly purified preparation of the rat enzyme (10) recognized a cluster of cytochrome P450 proteins in rat liver extracts that migrated with molecular weights in the 50,000 range (lanes 1 and 2). Within this group of proteins, the 7 α -hydroxylase enzyme is known to have the slowest electrophoretic mobility (10). In liver extracts from wild-type mice, this antibody recognized two major proteins, one of which had

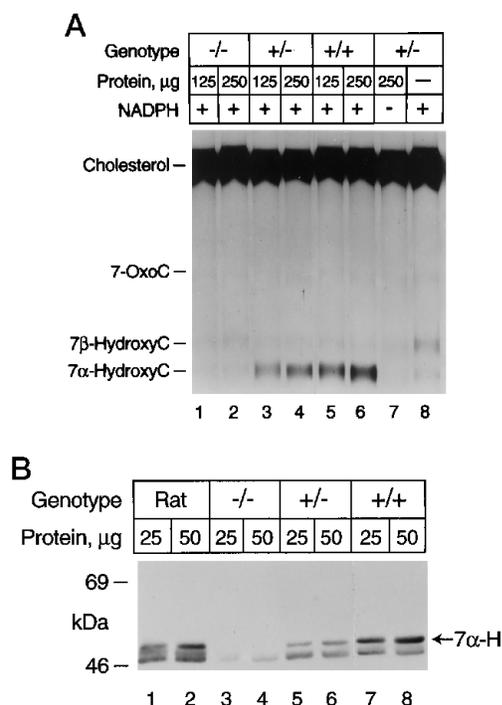


FIG. 2. Absence of 7 α -hydroxylase enzyme activity and protein in mutant mice. *A*, microsomal membranes were prepared from the livers of mice with the indicated genotype and assayed for 7 α -hydroxylase enzyme activity as described under "Experimental Procedures." An autoradiogram of a thin-layer chromatogram is shown. The positions to which the cholesterol substrate and the 7 α -hydroxycholesterol (7 α -HydroxyC) product of the enzyme migrated are indicated on the left. Also shown are the positions to which the spontaneous oxidation products 7-oxocholesterol (cholest-5-ene-7-oxo-3 β -ol) (7-OxoC) and 7 β -hydroxycholesterol (cholest-5-ene-3 β ,7 β -diol) (7 β -HydroxyC) migrated. *B*, shown is the detection of 7 α -hydroxylase protein in normal and mutant animals by immunoblotting. The indicated amounts of microsomal protein isolated from rats or from mice of different *Cyp7* genotypes were electrophoresed on an SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with a polyclonal antibody that recognizes the 7 α -hydroxylase protein (7 α -H).

a molecular weight identical to that of the rat 7 α -hydroxylase enzyme (lanes 7 and 8). The intensity of this putative murine 7 α -hydroxylase protein in liver extracts prepared from *Cyp7*^{+/-} animals (lanes 5 and 6) was approximately one-half that detected in normal mice, while this protein was not detected in *Cyp7*^{-/-} animals (lanes 3 and 4). The intensity of the other major cross-reacting protein, a presumed cytochrome P450 that has a lower molecular weight than the 7 α -hydroxylase protein, also declined in the homozygous animals (lanes 3 and 4). This decline appeared to be due to an overall decrease in the content of cytochrome P450 enzymes present in the livers of *Cyp7*^{-/-} mice (see below).

RNA blotting of poly(A)⁺-enriched mRNA fractions from mice of different *Cyp7* genotypes was performed next. As illustrated by the data of Fig. 3, wild-type mice contain several cholesterol 7 α -hydroxylase mRNAs, with major species at ~4.4 and ~7.0 kb. Heterozygous animals appear to contain slightly more of these same mRNAs and, in addition, a new mRNA migrating with a size of ~5.0 kb. *Cyp7*^{-/-} animals contain the latter mRNA, but none of the mRNA species present in wild-type mice. RNA blotting, cDNA cloning, and DNA sequencing experiments revealed that the novel mRNA present in the *Cyp7*^{+/-} and *Cyp7*^{-/-} animals was a hybrid mRNA composed of sequences derived from the 5'- and 3'-ends of the mutated *Cyp7* gene and the inserted neomycin resistance gene (data not shown). Control RNA blotting experiments (Fig. 3, lower panel) showed that the levels of sterol 27-hydroxylase mRNA and

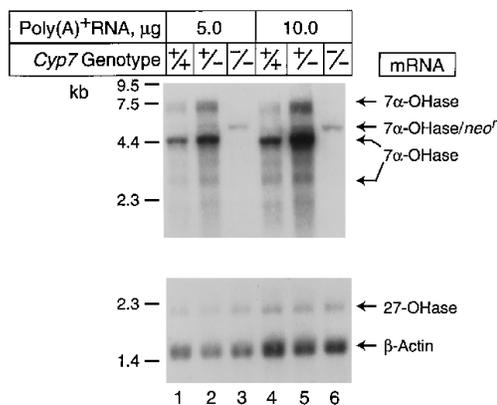


FIG. 3. RNA blotting in wild-type and 7 α -hydroxylase-deficient mice. Poly(A)⁺-enriched RNA was isolated from the livers of mice with the indicated *Cyp7* genotype, and 5.0- μ g aliquots (lanes 1–3) or 10- μ g aliquots (lanes 4–6) were subjected to blot hybridization. In the upper panel, the filter was probed with a radiolabeled 7 α -hydroxylase (7 α -OHase) cDNA. Several species of hybridizing mRNA were detected, and these are labeled on the right. In the lower panel, the same filter was stripped and re-probed with radiolabeled cDNAs for sterol 27-hydroxylase (27-OHase) and β -actin mRNAs. Size standards are indicated on the left. The exposure time was 16 h for both panels.

β -actin mRNA in the liver did not differ between wild-type and mutant *Cyp7* mice.

A phenotype associated with the absence of 7 α -hydroxylase became apparent upon breeding heterozygous animals. Homozygous pups arising from these crosses failed to thrive and experienced a very high rate of perinatal mortality. We hypothesized that death was caused by a deficiency of bile acids and a subsequent failure to absorb fat-soluble vitamins from the diet. To test this hypothesis, nursing mothers were placed on diets supplemented with vitamins and cholic acid as described under "Experimental Procedures." The survival rate of the *Cyp7*^{-/-} animals increased dramatically upon dietary supplementation for a period of 30 days. Thereafter, the mutant animals could be switched to normal chow with no decrease in survival. Using this supplementation regime, the mutant mouse colony was expanded to produce sufficient animals to characterize in detail their phenotype and the effects of individual dietary supplements.

We first determined the survival rate of animals born to *Cyp7*^{-/-} mothers in the absence of supplementation. As shown in Fig. 4A, ~85% of the pups arising from crosses of homozygous animals died within the first 18 days of postnatal life. Death occurred in two temporal waves, with ~40% of the pups succumbing prior to day 4 and the remainder dying between days 11 and 17. The 10–15% of the homozygous animals that survived until day 18 showed a near normal survival rate over the following 12 days (Fig. 4A). In these experiments, all pups suckled regardless of genotype, and milk was visible in the stomachs and intestines of the animals. Thus, death could not be attributed to the absence of alimentation. Homozygous pups defecated clay-colored, fatty stools, indicative of fat malabsorption (steatorrhea).

The effects of individual dietary supplements on animal survival were determined next. As shown in Fig. 4B, the presence of vitamins in the water decreased the numbers of deaths that occurred in the first few days of life, but did not prevent the second wave of death. Conversely, cholic acid supplementation of the mother's diet had no effect on the number of early deaths, but prevented the second temporal wave of death in the homozygous pups (Fig. 4B).

The death curve of *Cyp7*^{-/-} pups nursed by mothers given both vitamins and cholic acid is shown in Fig. 4C. When the supplements were begun on the day the pups were born, ~60%

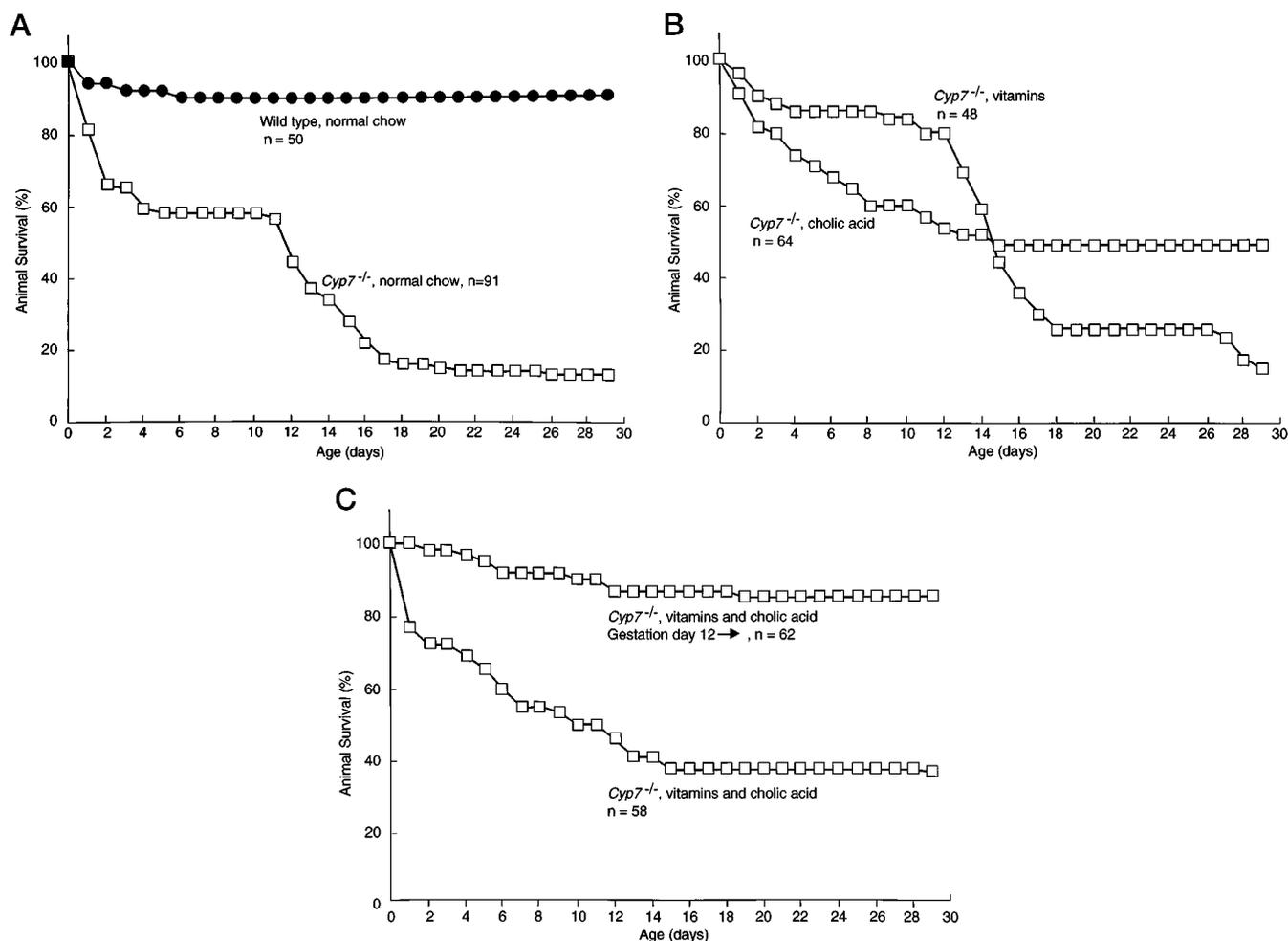


FIG. 4. **Survival of 7 α -hydroxylase-deficient mice.** Mice of the indicated genotypes were mated, and the survival of the resulting pups was determined. The results are plotted as a percentage of pups surviving per postnatal day of life. *A*, wild-type (●) or *Cyp7 α* ^{-/-} (□) nursing mothers were maintained on normal chow. *B*, *Cyp7 α* ^{-/-} mothers were maintained on chow supplemented with vitamins or 1% (w/w) cholic acid. *C*, *Cyp7 α* ^{-/-} mothers were maintained on chow supplemented with combinations of vitamins and 1% cholic acid beginning either at the time of birth (day 1) or on day 12 of gestation.

of the pups died during postnatal days 1–14. All of the animals that survived to day 15 remained alive at day 30. In contrast, when the dual supplementation was begun on gestation day 12 (*i.e.* before the pups were born), the survival rate of homozygous pups (Fig. 4C) was indistinguishable from that of wild-type pups born to mothers fed normal chow (Fig. 4A). Taken together, the results of the feeding studies indicated that 7 α -hydroxylase-deficient animals suffered from a vitamin shortage immediately after birth and from a bile acid shortage around the second week of life.

Mutant mice born to *Cyp7 α* ^{-/-} mothers maintained on normal chow were noticeably smaller than age-matched heterozygous or wild-type pups. To quantify this difference, weight gain in newborn animals was measured as a function of age and genotype. The data of Fig. 5 indicate that wild-type pups gained weight at an essentially linear rate during postnatal days 1–30. In contrast, homozygous pups gained weight at a slower rate until day 5 and then experienced a period of weight stagnation until day 11 (Fig. 5). After day 11, surviving mutant pups sustained a progressive weight gain, but at a slower rate compared with wild-type animals. A comparison of the death curve shown in Fig. 4 and the weight gain curve of the homozygous animals shown in Fig. 5 indicates that weight stagnation in the postnatal day 5–11 period was not accompanied by death of the pups. *Cyp7 α* ^{-/-} pups born to mothers whose diets were supple-

mented with vitamins and cholic acid during and after gestation gained weight at a normal rate (data not shown).

A second striking phenotype associated with disruption of the 7 α -hydroxylase gene was an abnormality of the fur. As shown in Fig. 6 (*upper panel*), nursing wild-type mothers have a glossy smooth pelt. In contrast, nursing homozygous mothers and, more surprisingly, nursing heterozygous mothers developed an unusually oily coat (a “Brylcream phenotype”) between days 4 and 7 of the nursing period (*center panel*). Approximately 3–4 days after a mother developed this phenotype, her pups also developed an oily hide (*lower panel*). In mothers, this phenotype persisted for a period of at least 7–8 days and as long as 14 days. The duration and extent of the symptom appeared to be directly related to the number of pups nursed. Females nursing larger litters exhibited a more severe phenotype for longer periods of time compared with mothers with smaller litters. Pups remained oily for ~7 days after the onset of symptoms, and most of them died within this period. In contrast, lactating females maintained on a 4% fat diet supplemented with 1% cholic acid did not develop the greasy phenotype, nor did the pups of these animals manifest the symptom. Vitamin supplementation alone had no effect on the development of the oily coat (data not shown).

During the nursing period, pups and young animals deficient in 7 α -hydroxylase also exhibited the phenotype shown in Fig. 7.

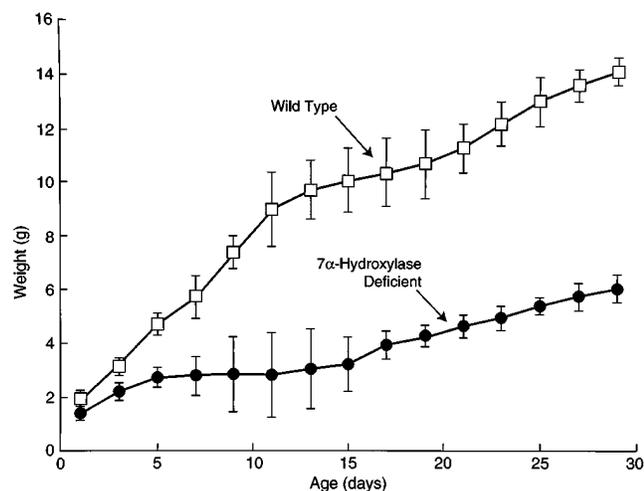


FIG. 5. **Weight gain in wild-type and 7 α -hydroxylase-deficient mice.** Newborn pups arising from crosses of wild-type or *Cyp7^{-/-}* deficient adults were weighed on a daily basis beginning on postnatal day 1 and proceeding through day 29. Average pup weight is plotted as a function of postnatal age for wild-type (\square) or homozygous deficient (\bullet) animals. Error bars represent mean \pm S.D. Weights were taken from a total of 22 wild-type pups arising from three litters and from 41 *Cyp7^{-/-}* pups arising from seven litters.

The photograph shows the skin of a day 5 pup, which is characterized by a dry, scaly appearance. This feature of the deficient mice is easiest to visualize before the growth of fur (Fig. 7); however, the symptom persists throughout the nursing period. In addition, older *Cyp7^{-/-}* animals showed eye abnormalities, including delayed opening and excess secretion emanating from the unopened oculi (data not shown). The eyelids of deficient animals opened \sim 8–9 days later than those of control mice.

The data of Fig. 8 show a histological analysis of the skin of wild-type and *Cyp7^{-/-}* pups. The *upper panel* represents an abdominal skin section of a day 14 wild-type animal that exhibited a normal histology. In contrast, the skin of an age-matched *Cyp7^{-/-}* pup that showed the scaly skin phenotype of Fig. 7 reveals a thickened and torn stratum corneum layer (hyperkeratosis) and a thickened granular layer (*lower panel*). The cells of the spinous and basal layers of the epidermis as well as the dermis of the homozygous animals appeared compressed relative to the wild-type specimen.

Liver histology in the deficient animals was normal, with well formed lobules and alternating central vein and portal triad architecture (data not shown). Yellow crystalline deposits were observed in the bile canaliculi of young mice. The level of conjugated bilirubin was higher in the livers of *Cyp7^{-/-}* mice (28.2 ± 2.8 μ g/mg tissue, $n = 3$) than in age-matched wild-type controls (2.6 μ g/mg ± 0.5 , $n = 3$). Finally, spectrophotometric scans of liver membrane proteins revealed an \sim 50% decrease in A_{450} absorbing material in the deficient *versus* wild-type animals (data not shown). These results, which indicate a decrease in liver cytochrome P450 content in the *Cyp7^{-/-}* mice, further suggest a disruption of heme metabolism in this organ.

Young *Cyp7^{-/-}* animals, after they had opened their eyes, appeared to be less active than wild-type counterparts. To confirm this observation, the feet of animals of different genotypes were dipped in blue ink (wild-type) or red ink (*Cyp7^{-/-}*), and the mice were placed in the upper right-hand corner of an enclosed sheet of filter paper (46 \times 57 cm). The mice were then allowed to roam undisturbed for 1 min. This procedure was repeated 11 additional times for each mouse. The wild-type mouse actively explored the enclosure as indicated by the presence of blue footprints over much of the paper (Fig. 9). The



FIG. 6. **Brylcream phenotype of nursing mothers and pups deficient in 7 α -hydroxylase.** A normal lactating female is shown in the *upper panel*. Note the smooth glossy appearance of the coat. A lactating *Cyp7^{-/-}* female is shown in the *center panel*. The coat is oily, and the hair is clumped into spikes. A group of three *Cyp7^{-/-}* pups photographed on postnatal day 14 exhibiting oily coats is shown in the *lower panel*.

majority of the movements of the wild-type mouse were confined to the perimeter of the enclosure, but the animal also crossed over the center of the paper. The concentration of blue footprints in each of the four corners reflects the animal's attempts to peer over and to climb out of the enclosure. In contrast to these results, a *Cyp7^{-/-}* animal confined its movements to one side of the paper, persistently ambling up and down the wall adjacent to the corner in which it was placed (Fig. 9, *red footprints*). This behavior suggests that the *Cyp7^{-/-}*



FIG. 7. **Abnormal skin phenotype in 7 α -hydroxylase-deficient pups.** The characteristic flaky skin of a postnatal day 5 *Cyp7*^{-/-} pup is shown.

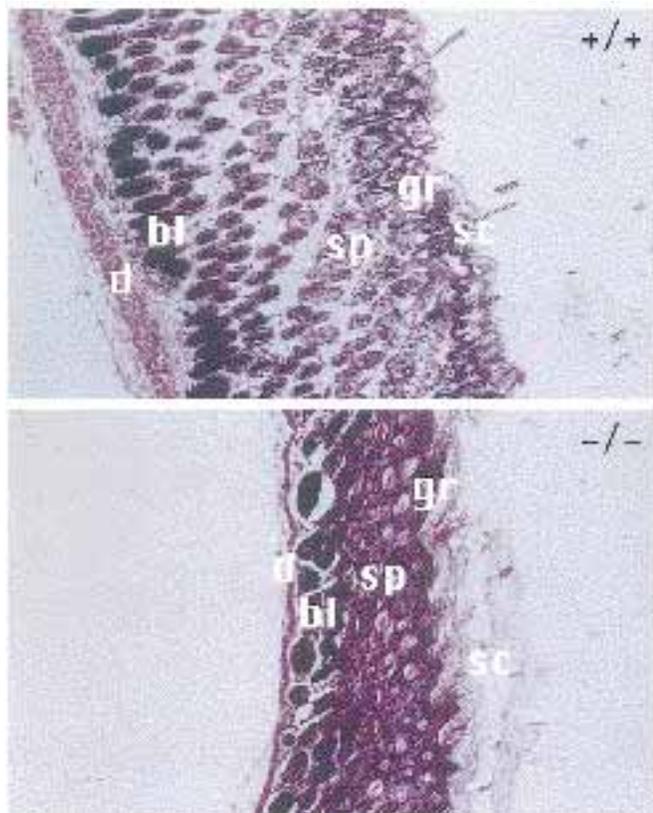


FIG. 8. **Skin histology of wild-type and 7 α -hydroxylase-deficient mice.** The *upper panel* shows a section of skin from the abdomen of a wild-type adult animal (14 days old) stained with hematoxylin and eosin. Cells of the stratum corneum (*sc*), granular layer (*gr*), spinous layers (*sp*), basal layer (*bl*), and dermis (*d*) are indicated. The *lower panel* shows a section derived from an age-matched *Cyp7*^{-/-} mouse. Note the thickened and blistered appearance of the stratum corneum and the overall condensed appearance of the specimen.

mice have vision problems that render them dependent on tactile stimulation for location cues and subsequent movement.

DISCUSSION

We provide evidence that 7 α -hydroxylase activity is important in murine postnatal life. The majority of mice that lack this enzyme die within 18 days of birth. Death is preceded by abnormally slow weight gain, fatty stools, hyperkeratosis, an

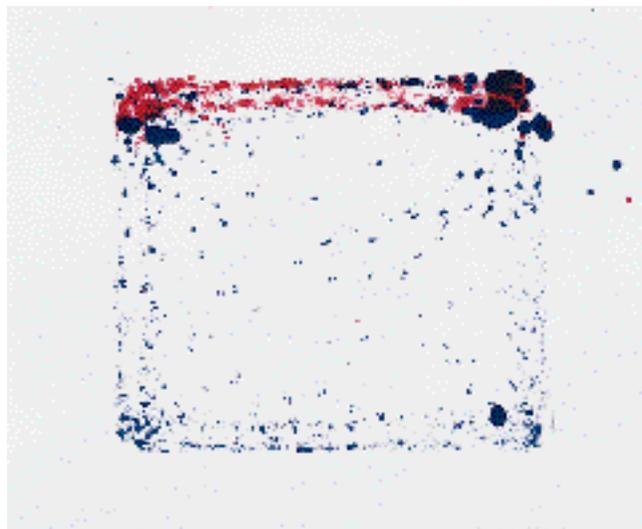


FIG. 9. **Open field test of wild-type and 7 α -hydroxylase-deficient mice.** The feet of a wild-type mouse were dipped in blue ink, and the animal was placed in the upper right-hand corner of a sheet of filter paper and allowed to roam freely for a period of 1 min. A similar protocol was followed for an age-matched *Cyp7*^{-/-} mouse, except the feet were dipped in red ink. The inking of the feet and placement of the animals on the paper were repeated 11 additional times to derive the composite shown.

unusual appearance of the fur, and behavioral abnormalities. These symptoms are representative of those associated with fat malabsorption (wasting and steatorrhea) and fat-soluble vitamin malabsorption (skin, fur, and vision pathologies), and their occurrence in *Cyp7*^{-/-} mice suggests that the bile acid pathway initiated in the endoplasmic reticulum by 7 α -hydroxylase is crucial for nutritional homeostasis in the neonate.

No decrease in fecundity (litter size) or effects on fertility were detected in *Cyp7*^{-/-} females, suggesting that 7 α -hydroxylase is not required for embryonic development or reproduction. At birth, 7 α -hydroxylase-deficient mice appear phenotypically normal. However, shortly thereafter, many of the homozygous animals begin to succumb to the deficiency. Deaths cluster in two time periods, days 1–4 and days 12–17 (Fig. 4A). Animals that survive through day 4 remain alive until day 12, at which time another 40% expire during the second period of increased death. The animals that survive through day 17 (~15% of the total) thereafter have lifespans that approximate those of wild-type mice.

Dietary supplementation experiments provide a potential explanation for the observed biphasic death curve. Pups born to mothers supplemented with a combination of fat- and water-soluble vitamins survive the first wave of early postnatal demise, but succumb to the second (Fig. 4B). In these experiments, vitamins were added to the mothers' water supply, which the nursing pups cannot reach and thus do not have direct access to. Given this mode of delivery, the simplest interpretation of the rescue data is that the early deaths are a consequence of a vitamin deficiency due to a decrease in the vitamin content of the mother's milk. The metabolism of postpartum animals changes radically with the onset of lactation since vastly more nutritional resources must be committed to the production of milk. The stress imparted by these metabolic changes is presumably revealing a phenotype (avitaminosis) in the deficient mothers that is otherwise not detectable in non-nursing females. If this interpretation is correct, then the physiological stress imparted to the *Cyp7*^{-/-} mothers by lactation and nursing overwhelms their capacity to solubilize sufficient vitamins to meet the needs of the pups. The resulting low levels of vitamins in the mother's milk, combined with the pups'

deficiency in bile acids, leads to the failure of the pups to absorb sufficient vitamins to maintain survival. Supplementation of the mother provides high enough levels of vitamins to the pups such that a sufficient amount can be absorbed to stave off death.

In the accompanying paper (15), we show that *Cyp7^{-/-}* mice are deficient in vitamins D₃ and E; thus, it seems likely that deficiency in one or more of the fat-soluble vitamins underlies the first wave of postnatal death. This deduction is in part based on the known role of bile acids in facilitating the uptake of fat-soluble vitamins (1) and the fact that mice lacking apolipoprotein B expression in the intestine, which are defective in fat-soluble vitamin uptake, also die at a high rate in early postnatal life (16). Alternatively, the phenotype of the 7 α -hydroxylase-deficient mice may reflect a deficiency of both water- and fat-soluble classes of vitamins in that the malabsorption accompanying the defect could lead to a generalized loss of vitamins. The phenotype of the *Cyp7^{-/-}* mice does not match the symptoms associated with a deficiency of any single water-soluble vitamin in the mouse (17), except perhaps folic acid. This vitamin is moderately soluble in water (1.6 μ g/ml at 25 °C), and a deficiency in the mouse leads to weight loss and the appearance of an "untidy hair coat" (18), which resembles that of the 7 α -hydroxylase-deficient animals (Fig. 6). We are carrying out supplementation studies in which individual vitamin classes or vitamins are given to the deficient animals in an attempt to identify the active substance in the current pan supplement.

An unanswered question that arises from the feeding experiments is why supplementation with vitamins and bile acids beginning on postnatal day 1 did not prevent deaths in the initial period (Fig. 4C), whereas supplementation with vitamins alone did (Fig. 4B). A generalized toxicity of the combined supplements can be ruled out since their administration during gestation increased pup survival (Fig. 4C). These results suggest that the metabolism, maternal-fetal transfer, or uptake of bile acids or vitamins changes in some as yet undefined manner at term or shortly thereafter.

Pups born to mothers fed a chow diet supplemented with 1% cholic acid die at the expected rate during the first lethal period, but survive the second wave of death (Fig. 4B). Death during the second period, but not the first, is preceded by the development of an oily coat in unsupplemented nursing mothers and their pups. In the vitamin supplementation experiments, we are certain that the pups did not gain direct access to the water bottles containing vitamins. However, in the case of the cholic acid supplementation experiments, the supplemented chow was strewn about the cage and adhered to the fur of both mothers and pups. Since the pups groom themselves, they may have consumed some of the cholic acid present in the chow. Thus, reversal of the second wave of death may have been due to alleviation of a maternal defect or an offspring defect associated with bile acid deficiency. A clue to under-

standing why the deficient mice die during postnatal days 11–17 may lie in determining the cause of the oily coat as cholic acid-supplemented animals did not die and did not develop this phenotype.

We initially hypothesized that the second period of death might be due to the deficiency of an essential fatty acid. Diets lacking in these fats (linolenic acid and linoleic acid) give rise to hyperkeratosis and excess water loss in mice (19), which is a phenotype that resembles the deficient mice of this study. However, as indicated in the accompanying paper (15), 7 α -hydroxylase-deficient mice have near normal levels of essential fatty acids. Thus, the second period of death may result from the accumulation of a toxic intermediate in the bile acid pathway, a secondary sterol, or a lipid metabolite in the *Cyp7^{-/-}* pups and their mothers. Alternatively, the animals may die of malnutrition caused by a failure to solubilize milk fats during the first 18 days of life.

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