Impaired Retinol Utilization in *Adh4* Alcohol Dehydrogenase Mutant Mice

LOUISE DELTOUR,† MARIO H. FOGLIO, AND GREGG DUESTER*
Gene Regulation Program, Burnham Institute, La Jolla, California

ABSTRACT *Adh4*, a member of the mouse alcohol dehydrogenase (ADH) gene family, encodes an enzyme that functions in vitro as a retinol dehydrogenase in the conversion of retinol to retinoic acid, an important developmental signaling molecule. To explore the role of *Adh4* in retinoid signaling in vivo, gene targeting was used to create a null mutation at the *Adh4* locus. Homozygous *Adh4* mutant mice were viable and fertile and demonstrated no obvious defects when maintained on a standard mouse diet. However, when subjected to vitamin A deficiency during gestation, *Adh4* mutant mice demonstrated a higher number of stillbirths than did wild-type mice. The proportion of liveborn second generation vitamin A-deficient newborn mice was only 15% for *Adh4* mutant mice but 49% for wild-type mice. After retinol administration to vitamin A-deficient dams in order to rescue embryonic development, *Adh4* mutant mice demonstrated a higher resorption rate at stage E12.5 (69%), compared with wild-type mice (30%). The relative ability of *Adh4* mutant and wild-type mice to metabolize retinol to retinoic acid was measured after administration of a 100-mg/kg dose of retinol. Whereas kidney retinoic acid levels were below the level of detection in all vehicle-treated mice (<1 pmol/g), retinol treatment resulted in very high kidney retinoic acid levels in wild-type mice (273 pmol/g) but 8-fold lower levels in *Adh4* mutant mice (32 pmol/g), indicating a defect in metabolism of retinol to retinoic acid. These findings demonstrate that another retinol dehydrogenase can compensate for a lack of *Adh4* when vitamin A is sufficient, but that *Adh4* helps optimize retinol utilization under conditions of both retinol deficiency and excess. Dev. Genet. 25:1–10, 1999.

Key words: alcohol dehydrogenase; retinol dehydrogenase; gene targeting; retinol; retinoic acid; vitamin A deficiency

INTRODUCTION

Vitamin A (retinol) regulates vertebrate growth and development via its metabolite retinoic acid, which functions as a ligand controlling a nuclear receptor signaling pathway [reviewed in Kastner *et al.*, 1994; Mangelsdorf *et al.*, 1994]. The actions of retinoic acid are mediated by several retinoic acid receptors capable of regulating transcription in response to binding of retinoic acid. Many of the functions of these receptors have been clearly established in retinoid receptor gene knockout mice, which display the classical defects of vitamin A deficiency, as well as additional defects in growth and development [Lohnes *et al.*, 1994; Mendelsohn *et al.*, 1994; Luo *et al.*, 1996]. A major challenge that remains in the study of vitamin A function is the delineation of how retinoid metabolism is normally controlled to provide the ligand for this signaling pathway.

Vitamin A is enzymatically converted to retinoic acid by a two-step pathway in which retinol is first oxidized to retinal, followed by oxidation of retinal to retinoic acid [reviewed in Duester, 1996]. Enzymes capable of catalyzing the oxidation of retinol to retinal in vitro fall into either the alcohol dehydrogenase (ADH) family containing members that function as cytosolic retinol dehydrogenases [Bliss, 1951; Zachman and Olson, 1961; Boleda *et al.*, 1993; Yang *et al.*, 1994; Allali-Hassani *et al.*, 1998; Han *et al.*, 1998; Kedishvili *et al.*, 1998] or the short-chain dehydrogenase/reductase (SDR) family, which has members that function as microsomal retinol dehydrogenases [Simon *et al.*, 1995; Chai *et al.*, 1995; Mertz *et al.*, 1997; Gough *et al.*, 1998]. The second step, oxidation of retinal to retinoic acid, is catalyzed by members of the aldehyde dehydrogenase (ALDH) family [Lee *et al.*, 1991; Labrecque *et al.*, 1995; Zhao *et al.*, 1996; Wang *et al.*, 1996; Ang and Duester, 1999]. Ongoing studies are aimed at determining which members of the ADH, SDR, and ALDH enzyme families

Contract grant sponsor: National Institutes of Health; Contract grant number: AA09731; Contract grant sponsor: National Cancer Institute.

†L. Deltour's present address is Institut Cochin de Genetique Moleculaire, Institut National de la Sante et de la Recherche Medicale U257, 24 rue du Faubourg Saint Jacques, 75014 Paris, France.

*Correspondence to: Gregg Duester, Gene Regulation Program, Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037. E-mail: duester@burnham-inst.org

Received 13 October 1998; Accepted 5 December 1998

participate in retinoid metabolism in vivo, as well as the nature of their particular physiological roles.

The vertebrate ADH family consists of at least seven distinct classes of cytosolic, zinc-dependent enzymes distinguished by sequence homology, enzymatic properties, and gene expression patterns [reviewed in Jörnvall *et al.*, 1995; Duester, 1996]. Most studies have focused on three forms—class I ADH (ADH1), class III ADH (ADH3), and class IV ADH (ADH4)—which are highly conserved in all mammalian species examined [ADH nomenclature reviewed in Duester *et al.*, 1999]. In the mouse, genes for all three of these ADHs have been identified (*Adh1, Adh3,* and *Adh4*) [Holmes, 1977; Zgombic-Knight *et al.*, 1995], and a retinol dehydrogenase isolated from mouse epidermis was identified as ADH4 [Connor and Smit, 1987]. In humans, ADH4 demonstrates higher retinol dehydrogenase activity than ADH1, with ADH1 having higher ethanol dehydrogenase activity than that displayed by ADH4, and with ADH3 having no detectable retinol dehydrogenase activity and very low ethanol dehydrogenase activity [Yang *et al.*, 1994; Allali-Hassani *et al.*, 1998; Han *et al.*, 1998; Kedishvili *et al.*, 1998]. Molecular modeling studies of human ADH4 based on X-ray crystal structure data have shown that it possesses a large active site that can easily accommodate retinol [Kedishvili *et al.*, 1995; Moreno *et al.*, 1996; Xie *et al.*, 1997]. In the mouse, ADH4 expression has been localized in numerous retinoid-responsive epithelia, including the testis/epididymis [Deltour *et al.*, 1997], epidermis [Haselbeck *et al.*, 1997b], and stomach/esophagus [Haselbeck and Duester, 1997], as well as in stage E8.5–E9.5 embryos during neurulation [Ang *et al.*, 1996a; Haselbeck and Duester, 1998b]. This finding suggests that ADH4 plays a role in local retinoic acid synthesis for autocrine or paracrine retinoid signaling. In addition, ADH4 and retinoic acid have been colocalized in the adult and embryonic adrenal gland, suggesting that this enzyme may also participate in the production of retinoic acid in this gland [Haselbeck *et al.*, 1997a; Haselbeck and Duester, 1998a].

Genetic studies have proved quite useful in the analysis of certain aspects of retinoid signaling, particularly the function of the retinoid receptors. However, genetic studies on the enzymes involved in retinoic acid synthesis have not been reported. In order to evaluate the role of ADH4 in retinoid signaling, we have generated mice carrying a knockout mutation of the *Adh4* gene. The results provide evidence that *Adh4* does indeed play a role in retinol utilization.

MATERIALS AND METHODS

Engineering of the *Adh4* Targeting Vector

The gene replacement targeting vector was produced as follows. A genomic clone of mouse *Adh4* (*Adh3* in old nomenclature) encoding class IV ADH from strain 129/SvJ has been described [Zgombic-Knight *et al.*, 1997]. A 2.5-kb *Bam*HI–*Xba*I DNA fragment containing exons 7 and 8 of *Adh4* (downstream homology) was subcloned into the *Sma*I site of pBluescript II KS, then excised with *Sal*I and *Xba*I and inserted into the corresponding sites of plasmid pTK-neo [Scott *et al.*, 1994] between the PGK-neo and PGK-tk gene cassettes to form pTK-neo-286. A 6.3-kb *Xba*I DNA fragment containing the 5′-flanking region of *Adh4* (upstream homology) was subcloned into the *Xba*I site of pBluescript II KS, then excised with *Xho*I and *Not*I and inserted between those sites downstream of PGK-neo in pTK-neo-286 to produce the *Adh4* gene targeting vector.

Generation of *Adh4* Null Mice

The *Adh4* gene targeting vector was linearized with *Not*I and introduced by electroporation into mouse embryonic stem cells (R1 cells from 129/Sv strain) using established methodology [Joyner, 1993]. Cells were subjected to both positive selection with G418 and negative selection with gancyclovir to enrich for cells incorporating the construct by homologous recombination. Genomic DNA was isolated from surviving cell clones and screened by Southern blot analysis [Hogan *et al.*, 1994] using *Hin*dIII digestion to identify cells in which a portion of the *Adh4* gene was deleted. The external DNA probe for Southern blot screening consisted of a 1.5-kb *Xba*I fragment containing exon 9 of *Adh4*. In *Adh4* +/− clones, the wild-type *Hin*dIII fragment was 4.0 kb, and the mutant *Hin*dIII fragment was 5.8 kb. The truncated mutant *Adh4* gene lacks the promoter and exons 1–6 containing the coding region for amino acid residues 1–275. Karyotype analysis was performed on Southern blot-positive clones to identify clones having a normal karyotype for further use.

Adh4 +/− mutant embryonic stem cells were microinjected into C57B1/6 blastocysts, which were then implanted into pseudopregnant females [Joyner, 1993]. This resulted in chimeric mice having a representation of cells derived from the 129/Sv embryonic stems cells as identified by patches of hair with the dominant agouti coat color of the 129/Sv strain. Several chimeric male mice were mated to wild-type female Black Swiss mice and germline transmission of the introduced embryonic stem cells was identified by offspring with a complete agouti coat color. Southern blot analysis of tail DNA [Hogan *et al.*, 1994] from agouti offspring was used to identify individuals heterozygous for the *Adh4* mutation, i.e., carrying both the 4.0- and the 5.8-kb *Hin*dIII fragments described above. Heterozygous *Adh4* +/− mutant mice were mated to produce homozygous *Adh4* −/− mutant mice also identified by Southern blot

analysis of tail DNA. *Adh4* mutant and wild-type mice were maintained on Purina basal diet 5755, unless otherwise stated.

Northern blot analysis with an *Adh4* cDNA probe was performed on mouse tissues using 10 μg of total RNA as previously described [Zgombic-Knight *et al.*, 1995]. Western blotting with polyclonal antibodies against mouse ADH1, ADH3, and ADH4 was accomplished using 20 μg of total protein from mouse tissues as reported [Haselbeck and Duester, 1997].

Staging of Embryos

The day of pregnancy and stage of embryonic development were determined by vaginal plug appearance, with noon on the day of plug detection considered embryonic day 0.5 (E0.5). Further staging of mouse embryos was according to Kaufman [1992].

Production of Vitamin A-Deficient Mice

Vitamin A deficiency was induced in mice (*Adh4* mutant and wild-type) by a modification of previous procedures [De Luca *et al.*, 1989; Smith *et al.*, 1987; Morriss-Kay and Sokolova, 1996]. Original parental female mice were fed Purina vitamin A-deficient diet 5822 (<0.22 IU/g vitamin A) for 16 weeks. Male mice used for all matings were maintained on vitamin A-sufficient Purina basal diet 5755 (22 IU/g vitamin A as retinyl acetate) when not being used for mating. Females subjected to vitamin A deficiency for 16 weeks were mated to produce vitamin A-deficient F_1 offspring maintained from birth on the deficient diet Purina 5822. Confirmation of vitamin A deficiency in F_1 offspring was obtained by observation of a reduction in weight gain following birth relative to offspring of dams maintained on the sufficient diet Purina 5755; also, unlike mice maintained on a sufficient diet, vitamin A-deficient F_1 mice had a high incidence of death starting at 9 weeks of age. Mating of 5-week-old vitamin A-deficient F_1 female mice produced vitamin A deficient F_2 progeny which were examined at birth in order to monitor the incidence of stillbirth (newborns that were not delivered naturally by stage E19.5 were delivered by cesarean section at E19.5).

Retinol Rescue of Vitamin A-Deficient Mouse Embryos

In order to establish cohorts of vitamin A-deficient mice (*Adh4* mutant and wild-type) in good health, vitamin A-deficient F_1 female mice were placed at 9 weeks of age on Purina vitamin A-deficient diet 5822 containing a low dose of retinoic acid (3 μg/g). This dose of retinoic acid has previously been shown to maintain the health of adult mice that are otherwise deficient for retinol or retinyl esters [De Luca *et al.*, 1989]. Mice were kept on the retinoic acid-containing 5822 diet for 4 weeks, then cycled back to the normal 5822 diet for 2 weeks to further deplete vitamin A, and finally back to the retinoic acid-containing 5822 diet to maintain health for at least 2 weeks before carrying out further studies. Studies on the ability of such female mice to reproduce were carried out by a modification of methods used for vitamin A-deficient female rats maintained on a retinoic acid-containing diet [Wellik and DeLuca, 1995]. Such rats are able to become pregnant but invariably resorb the embryos by stage E15.5 unless given a 2-μg dose of retinol by stage E10.5 [Wellik and DeLuca, 1995]. In order to perform this retinol rescue experiment in the vitamin A-deficient retinoic acid-supplemented female mice prepared above, we took into account their smaller weight and shorter gestation period relative to the rat and executed the following procedure. Mice were mated and at stage E8.5 of gestation the dams were administered by oral intubation a 0.1-ml dose containing 0.4 μg retinol, prepared by dilution of an ethanolic stock solution into corn oil. Dissection of retinol-treated pregnant mice was performed at stage E12.5 of gestation to monitor the progress of embryonic development and incidence of resorption.

Measurement of Retinoic Acid After Retinol Treatment

Methods for preparation of the retinol solution (20 mg/ml) and administration to adult mice by oral intubation have been previously described [Collins *et al.*, 1992]. The vehicle for retinol administration corresponded to acetone–Tween 20–water (0.25:5:4.75). All-*trans*-retinol was purchased from Sigma Chemical Co. (St. Louis, MO). Retinol (100 mg/kg) or vehicle control were administered at 5 μl/g of body weight by oral intubation to full-grown adult female mice of comparable age and weight. Two hours after administration of retinol or vehicle, tissues were dissected and retinoic acid was monitored qualitatively in tissue explants using a *lac*Z reporter cell bioassay as previously described [Ang *et al.*, 1996a; Haselbeck *et al.*, 1997a]. The reporter cells detect the sum of all active carboxylated retinoids including all-*trans*-retinoic acid by induction of β-galactosidase activity. Quantitation of retinoic acid levels in kidney homogenates was performed using a spectrophotometric variation of the reporter cell bioassay with all-*trans*-retinoic acid (Sigma) as the standard [Ang *et al.*, 1996b; Deltour *et al.*, 1997; Haselbeck *et al.*, 1997a]. The assay was performed essentially as described [Ang *et al.*, 1996b], except that tissue was homogenized for 30 seconds with a Tissue-Tearor homogenizer (Biospec Products, Inc.), followed immediately by centrifugation and application of the supernatant fraction to the reporter cells.

Statistical Methods

Statistical significance was determined for raw data using Fisher's exact test (two-sided) for the independence of two variables, or using the unpaired *t*-test

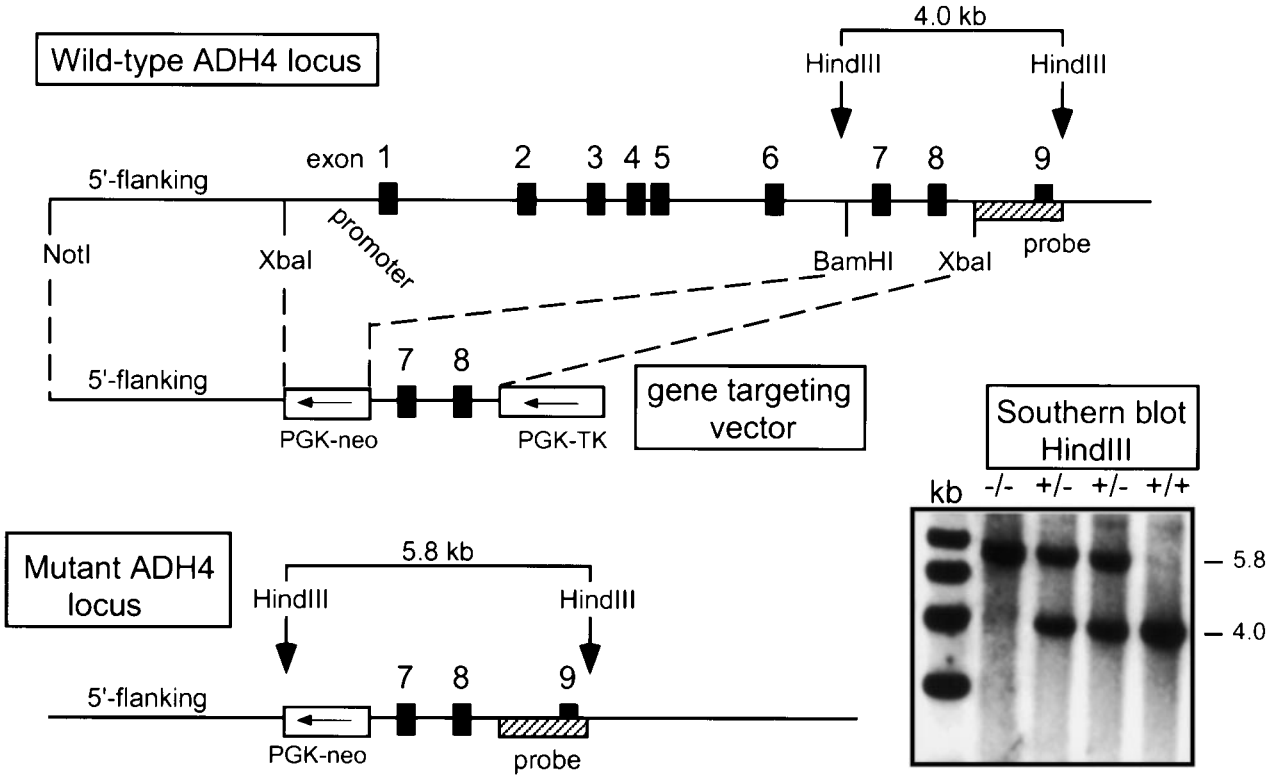

Fig. 1. Gene targeting of the *Adh4* locus. The wild-type Adh4 gene is shown indicating the nine exons as black boxes, a 3′ probe used for Southern blot analysis as a hatched box, and a 3′ *Hin*dIII fragment of 4.0 kb. The gene targeting vector was engineered to contain the 5′-flanking region separated from exons 7 and 8 by the PGK-neo cassette for positive selection, with the PGK-tk cassette for negative selection located further downstream. After correct targeting resulting in a gene replacement event the mutant *Adh4* locus is shown, now with a 3′ *Hin*dIII fragment of 5.8 kb, using the 3′ probe. Also shown is a Southern blot of individuals derived from a heterozygous (+/−) mating which resulted in homozygous (−/−) mutant mice.

(two-tailed) for the difference between means (GraphPad Prism version 2.0b, GraphPad Software, San Diego, CA).

RESULTS

Generation of *Adh4* Mutant Mice

The targeting vector used to generate a knockout mutation in the *Adh4* gene is shown along with maps of the wild-type and mutant *Adh4* loci (Fig. 1). Briefly, a gene replacement vector was prepared in which a 6.3-kb DNA fragment containing the 5′-flanking region of *Adh4* was cloned downstream of the PGK-neo cassette in pTK-neo [Scott *et al.*, 1994] and a 2.5-kb DNA fragment containing exons 7 and 8 was cloned upstream of PGK-neo. Linearized vector was introduced into R1 embryonic stem cells, and individual colonies selected in the presence of G418 (positive selection) and gancyclovir (negative selection) were screened by Southern blot analysis, using an external probe containing exon 9 of *Adh4*. After analysis of 101 independent clones, we retrieved three carrying the expected heterozygous mutation (*Adh4* +/−) identified by the presence of a mutant 5.8-kb *Hin*dIII fragment, in addition to the wild-type 4.0-kb fragment (Fig. 1). Karyotype analysis of these three clones identified one with a normal karyotype that was used for introduction into mice.

After introduction of *Adh4* +/− mutant embryonic stem cells into blastocysts, we recovered six male chimeric mice, four of which transmitted the mutation to their offspring. Crossing of heterozygous *Adh4* +/− mutant mice resulted in the production of homozygous *Adh4* −/− mutant mice as determined by Southern blot analysis (Fig. 1). Homozygous and heterozygous *Adh4* mutant mice were obtained at close to the expected Mendelian ratio (+/+ = 29%, +/− = 50%, −/− = 21%; from 90 total progeny), and no gross defects were observed in any mutant mice. Homozygous *Adh4* mutant mice of both sexes were fertile, and homozygous females gave birth to normal-size litters, which displayed no obvious defects. Thus, a deletion mutation at the *Adh4* locus does not cause a significant reduction in prenatal or postnatal survival of mice raised under standard laboratory conditions. All further studies were focused on only the wild-type (+/+) or homozygous *Adh4* mutant (−/−) mice.

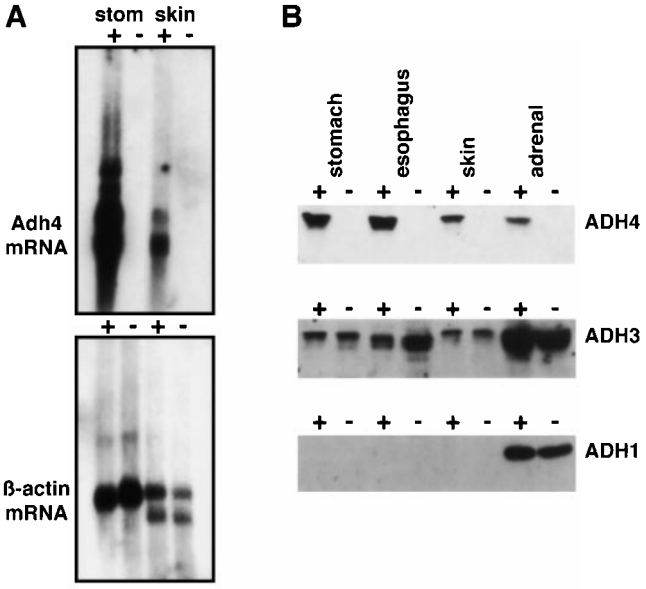

Fig. 2. Lack of *Adh4* expression in null mutant mice. **A:** Northern blot analysis of 10 µg stomach and skin RNA from *Adh4* −/− mutant mice (−) and *Adh4* +/+ wild-type mice (+) indicates a total lack of *Adh4* mRNA in the mutant. Integrity of the RNA samples is shown by detection of β-actin mRNA in both mutant and wild-type samples. **B:** Western blot examination of 20 µg total protein from stomach, esophagus, skin, and adrenal homogenates demonstrates a complete lack of ADH4 protein in −/− mutant mice (−) compared with +/+ wild-type mice (+). Levels of ADH3 and ADH1 protein are not affected by mutation of *Adh4*.

Lack of ADH4 Expression in Homozygous Mutant Mice

Northern blot analysis of stomach and skin RNA indicated that homozygous *Adh4* mutant mice have no detectable *Adh4* mRNA in either of these tissues, whereas wild-type mice have easily detectable *Adh4* mRNA (Fig. 2A). Western blot analysis indicated a lack of detectable ADH4 protein in stomach, esophagus, skin, and adrenal gland of *Adh4* mutant mice, compared with wild-type mice, which have easily detectable levels of ADH4 in all these tissues (Fig. 2B). Additional Western blot analysis of these same tissues with antibodies for two other mouse ADHs demonstrated normal levels of both ADH1 and ADH3 protein in *Adh4* mutant mice (Fig. 2B); previous studies have indicated that ADH3 is present ubiquitously, whereas ADH1 is present in adrenal, but not in the other tissues examined [Haselbeck and Duester, 1997]. These findings verify that the gene deletion resulted in a null mutation for *Adh4*, and had no effect on the expression of two other ADH genes, *Adh1* and *Adh3*.

Survival of *Adh4* Mutant Newborn Mice Subjected to Vitamin A Deficiency During Gestation

In order to explore the role of ADH4 in retinol utilization, *Adh4* mutant and wild-type mice were subjected to vitamin A deficiency as described in Materials and Methods. Females maintained on a vitamin A-deficient diet for 16 weeks were mated to produce vitamin A-deficient F_1 offspring, maintained from birth on the deficient diet. Vitamin A deficiency in female F_1 offspring was confirmed by observation of a reduction in weight gain at 5 weeks of age (*Adh4* mutant mean weight = 16.6 g; wild-type mean weight = 17.7 g) relative to F_1 offspring of females maintained on a sufficient diet (*Adh4* mutant mean weight = 22.4 g; wild-type mean weight = 21.6 g). However, no significant difference in litter size or growth and development was noticed between *Adh4* mutant and wild-type mice at the F_1 stage of vitamin A deficiency.

TABLE 1. Survival of Newborn *Adh4* Mutant and Wild-Type Mice Subjected to Vitamin A Deficiency During Gestation†

Genotype	Litters	Newborns	Newborns/litter	% Stillborn	% Liveborn
Adh4 +/+	6	41	6.8	51	49
Adh4 −/−	8	52	6.5	85	15*

†Original parental female mice were subjected to vitamin A deficiency (VAD) for 16 weeks, then mated to produce VAD F_1 dams used in this experiment. Mating of 5-week-old VAD F_1 dams produced the VAD F_2 progeny shown here examined at birth. See Fig. 3 for examples of stillborn and liveborn phenotypes.
*$P = 0.0006$, *Adh4* −/− vs. +/+ mice (Fisher's exact test).

To examine mice at a later stage of vitamin A deficiency, 5-week-old vitamin A-deficient F_1 female mice were mated to produce vitamin A-deficient F_2 progeny. At this stage of vitamin A deficiency, a significant difference in the survival of *Adh4* mutant and wild-type F_2 newborn mice was observed. Very few *Adh4* mutant progeny were delivered liveborn (most being stillborn), whereas a much higher percentage of wild-type progeny were delivered liveborn. The percentage of liveborn mice was only 15% for *Adh4* mutant mice, but 49% for wild-type mice (Table 1). Stillborn progeny appeared dead with wrinkled cyanotic skin indicating a lack of blood oxygenation, no movement, and no breathing (Fig. 3A). The phenotype of liveborn progeny was that of a normal newborn with smooth pink skin indicating blood oxygenation, movement of arms, legs, and mouth, and signs of breathing (Fig. 3B). Thus, although mutant and wild-type mice at the F_2 stage of vitamin A deficiency exhibited no difference in average litter size (6.5 for mutant and 6.8 for wild-type) or average newborn weight (1.06 g for mutant and 1.03 g for wild-type), mutation of *Adh4* led to reduced survival at the final stage of gestation.

Retinol Rescue of Vitamin A-Deficient *Adh4* Mutant Embryos

Unlike mice maintained on a sufficient diet, adult vitamin A-deficient F_1 mice (either *Adh4* mutant or wild-type) had a high incidence of death starting at 9

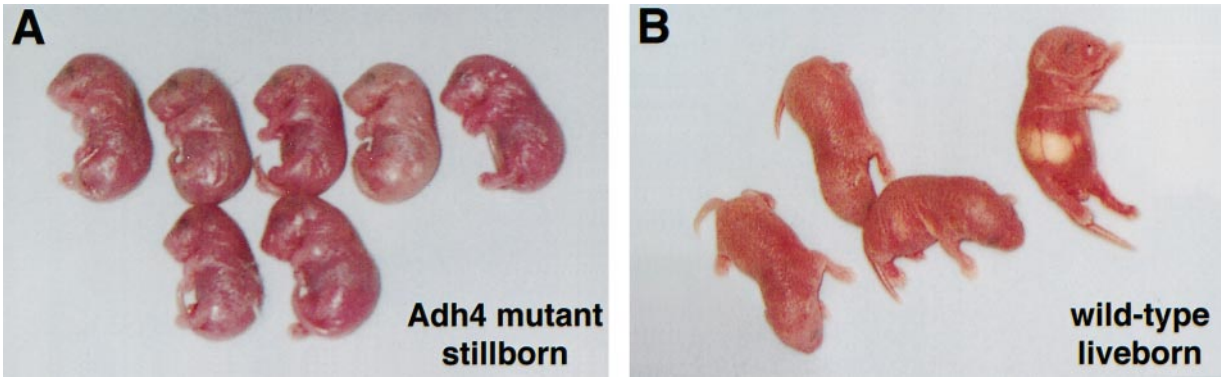

Fig. 3. Phenotypes of *Adh4* mutant and wild-type newborn mice during second generation vitamin A deficiency. **A:** Most *Adh4* mutant mice were stillborn as shown. The skin appeared wrinkled and was cyanotic indicating a lack of blood oxygenation. There was a lack of movement including an absence of breathing. **B:** Approximately one-half of the wild-type mice were liveborn as shown. The skin appeared smooth and was pink, indicating blood oxygenation. There was movement of arms, legs, and mouth, and signs of breathing.

TABLE 2. Rescue of *Adh4* Mutant and Wild-Type Stage E12.5 Embryos by Administration of Retinol to Vitamin A-Deficient Dams†

Genotype	Litters	Implantations	Implantations/ litter	% Implantations as embryos	% Implantations as resorptions
Adh4 +/+	5	30	6.0	70	30
Adh4 −/−	4	35	8.8	31	69*

†Parental vitamin A-deficient female mice of both genotypes were maintained on a diet containing a low dose of retinoic acid (3 μg/g). Dams were administered a dose of 0.4 μg retinol in corn oil by oral intubation on day 8.5 of gestation. Dissection was performed at day 12.5 of gestation.
*$P = 0.0028$, *Adh4* −/− vs. +/+ mice (Fisher's exact test).

weeks of age. In order to establish healthy colonies of vitamin A-deficient mice, vitamin A-deficient F_1 female mice were placed at 9 weeks of age on a vitamin A deficient diet supplemented with a small quantity of retinoic acid (3 μg/g). This amount of retinoic acid in the diet has been shown to maintain the health of vitamin A-deficient adult mice [De Luca *et al.*, 1989]. In rats, a similar diet has been shown to maintain adult health but is unable to support embryonic development past mid-gestation, resulting in resorption of all embryos, unless a dose of retinol is also administered by mid-gestation [Wellik and DeLuca, 1995]. We performed such a retinol rescue experiment on pregnant *Adh4* mutant or wild-type mice maintained on the vitamin A-deficient diet supplemented with retinoic acid. We administered a dose of 0.4 μg retinol to the dams at stage E8.5 of gestation, followed by dissection at stage E12.5 to monitor the progress of embryonic development and incidence of resorption. *Adh4* mutants exhibited a significantly higher resorption rate (69%) than did wild-type mice (30%) indicating a lower incidence of retinol rescue when ADH4 is missing (Table 2).

Effect of *Adh4* Mutation on Metabolism of Retinol to Retinoic Acid

Previous studies have shown that adult mice exposed to a large dose of retinol will metabolize most of it very quickly in an oxidative pathway that produces large amounts of retinoic acid which is then further oxidized [Collins *et al.*, 1992]. To address the possibility that ADH4 participates in this type of metabolism, retinol (100 mg/kg) or vehicle control were administered to adult female mice (*Adh4* mutant or wild-type) maintained on a normal basal diet. At 2 h after treatment, tissue explants from kidney, heart, and spleen were qualitatively monitored for the presence of retinoic acid using a reporter cell bioassay. The bioassay employs a retinoic acid-inducible *lacZ* gene and an in situ β-galactosidase detection method that can detect retinoic acid released from cultured tissues. We have previously shown (in untreated wild-type mice) that tissue explants from kidney, heart, and spleen grown on the reporter cells have undetectable levels of retinoic acid [Ang *et al.*, 1996b]. In the present experiment, vehicle-treated *Adh4* mutant or wild-type mice both had undetectable levels of retinoic acid in kidney, heart, or spleen tissue explants. However, treatment with retinol led to a high level of retinoic acid detection in all these tissues of wild-type mice but a significantly lower level of detection in *Adh4* mutant mice (data not shown for this qualitative assay). Quantitation of retinoic acid levels in treated *Adh4* mutant and wild-type mice was performed on kidney extracts using a spectrophotometric variation of the reporter cell bioassay [Ang *et al.*, 1996b]. Kidney retinoic acid levels in vehicle-treated *Adh4* mutant or wild-type mice were both below the

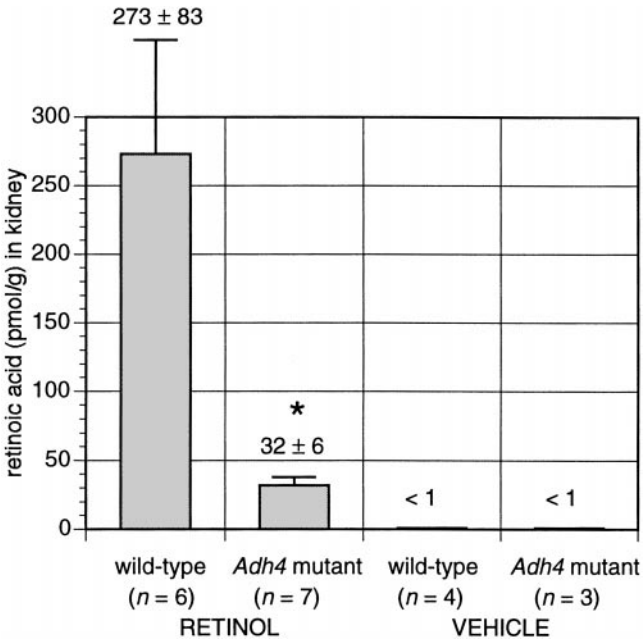

Fig. 4. Retinoic acid levels in kidneys of *Adh4* mutant and wild-type mice after retinol administration. Retinol (100 mg/kg) or vehicle control was administered by oral intubation. After 2 hours animals were sacrificed and retinoic acid levels were measured in kidney homogenates. The number of mice (*n*) treated is shown in parentheses. Values are presented as the mean ± S.E.M., except for vehicle-treated mice where the level was below the limit of detection for the assay (<1 pmol/g). *$P = 0.0089$, retinol-treated *Adh4* mutant vs wild-type mice (unpaired t-test).

limit of detection of the assay (<1 pmol/g). In retinol-treated wild-type mice, the kidney retinoic acid content rose dramatically to 273 ± 186 pmol/g, but in retinol-treated *Adh4* mutant mice, this value rose to only 32 ± 6 pmol/g, representing an 8-fold reduction in conversion of retinol to retinoic acid in the absence of ADH4 (Fig. 4).

DISCUSSION

Previous in vitro studies of mammalian ADH4 provided evidence that this enzyme is relatively inefficient in catalyzing the oxidation of ethanol compared with the classical liver enzyme ADH1, but that it is quite efficient for catalysis of retinol oxidation needed in the synthesis of retinoic acid [Allali-Hassani *et al.*, 1998; Han *et al.*, 1998; Kedishvili *et al.*, 1998]. Expression of *Adh4* mRNA and localization of ADH4 protein in several retinoid-responsive adult tissues of the mouse, such as epidermis, testis, and upper gastrointestinal tract, further support a role for this enzyme in retinoic acid synthesis [Haselbeck *et al.*, 1997b; Deltour *et al.*, 1997; Haselbeck and Duester, 1997]. *Adh4* is also expressed in mouse embryos from the neural fold stage onward, suggesting that it may play a role in local embryonic retinoic acid synthesis needed for growth and development [Ang *et al.*, 1996a; Haselbeck and Duester, 1998b]. A potential role for ADH4 in adrenal gland production of retinoic acid has been proposed on the basis of evidence of robust expression of *Adh4* mRNA and protein in the adult and embryonic adrenal glands, as well as high amounts of retinoic acid in this tissue [Haselbeck *et al.*, 1997a; Haselbeck and Duester, 1998a]. In the studies reported in this paper, we now provide in vivo evidence supporting a role for mouse ADH4 in retinol utilization.

Up to this point, there have been no genetic studies linking ADH4 or any other enzyme to retinoic synthesis. We generated a null mutation in mouse *Adh4* to address the question of whether ADH4 contributes to retinol utilization. *Adh4* mutant mice show no obvious phenotype when maintained under normal laboratory conditions on a standard mouse diet. This was not unexpected, owing to the likelihood of functional redundancy between ADH4, ADH1, and microsomal retinol dehydrogenases. Since it is clear that retinol is required for proper embryonic growth and development, most likely as a precursor for retinoic acid production [Wellik and DeLuca, 1995; Zile, 1998], we propagated *Adh4* mutant and wild-type mice under conditions of vitamin A deficiency, to determine whether at some point during the process of systemic retinol reduction mice missing a potential retinol dehydrogenase would be affected more severely than would wild-type mice. We found that second generation vitamin A-deficient *Adh4* mutant mice have a 3-fold lower survival rate at birth than wild-type mice presented with the same challenge. Under these conditions, most *Adh4* mutant newborns display a stillborn phenotype, indicating a block in development at the end of gestation. Previous studies in rats have shown that retinol is needed for neonatal survival [Wellik *et al.*, 1997]. Our findings indicate that the ability of retinol to function in neonatal survival is impaired when ADH4 is missing, implying that this enzyme facilitates the conversion of retinol to retinoic acid when vitamin A drops to dangerously low levels. This is consistent with the high catalytic activity of ADH4 as a retinol dehydrogenase relative to other enzymes [Allali-Hassani *et al.*, 1998; Han *et al.*, 1998; Kedishvili *et al.*, 1998].

We further examined the role of *Adh4* in retinol utilization by attempting to rescue embryonic development during vitamin A deficiency by administration of retinol. Previous studies have shown that vitamin A-deficient female rats maintained on a diet containing a low dose of retinoic acid can become pregnant, but that all embryos are resorbed by stage E15.5, unless a single small dose of retinol is administered to the dams by stage E10.5 [Wellik and DeLuca, 1995]. Since retinol is generally accepted as a prohormone unable to carry out signaling events on its own, the reason for its requirement by mid-gestation is presumably to function as a substrate that is metabolized to retinoic acid or some other active retinoid, which may be important

for maintenance of the entire embryo during gestation [Wellik and DeLuca, 1996]. We repeated this experimental system with pregnant vitamin A-deficient mice and found that mutation of *Adh4* reduces the ability of a small dose of retinol to rescue embryonic growth and development. Under these conditions, *Adh4* mutant embryos demonstrate a 2.3-fold higher incidence of resorption by stage E12.5, compared with wild-type embryos, indicating a reduction in retinol rescue in the absence of ADH4. These experiments show that ADH4 increases the efficiency of retinol utilization when this substrate is present at very low levels. It is unclear whether the utilization of retinol that occurs under these conditions is maternal or embryonic. If utilization by the embryo itself is critical at stage E12.5, it is likely that the embryonic adrenal gland is involved, since the primary site of *Adh4* expression at this stage is the developing adrenal blastema [Haselbeck and Duester, 1998a].

Studies on the metabolism of toxic doses of retinol in mice have previously shown that the major pathway for clearance involves oxidation to retinoic acid and that this pathway is reduced by inclusion of an ADH inhibitor [Collins *et al.,* 1992]. Retinoic acid, which is more toxic than retinol, is evidently cleared efficiently by further oxidation involving enzymes such as P450RAI [White *et al.,* 1996]. In our studies, we demonstrated that conversion of a toxic dose of retinol to retinoic acid is reduced 8-fold in tissues of *Adh4* mutant mice relative to wild-type mice. This provides direct evidence that ADH4 does participate in the oxidation of retinol to retinoic acid. Within the context of vitamin A toxicity, it can be proposed that the presence of ADH4 enhances retinol utilization, allowing the organism to clear itself of excess retinol which cannot be stored. Such a function for ADH4 may be important when the diet contains foods high in vitamin A, such as liver, or for individuals taking vitamin A supplements.

In addition to ADH4, the other enzymes reported to catalyze retinol oxidation include other cytosolic retinol dehydrogenases, such as ADH1 [Boleda *et al.,* 1993; Han *et al.,* 1998; Kedishvili *et al.,* 1998] or microsomal retinol dehydrogenases, which are members of the SDR enzyme family [Simon *et al.,* 1995; Chai *et al.,* 1995; Mertz *et al.,* 1997; Gough *et al.,* 1998]. It was initially proposed that the microsomal retinol dehydrogenases may be more specific for retinol than ADHs, but it was recently found that these enzymes actually function more efficiently as hydroxysteroid dehydrogenases than as retinol dehydrogenases [Biswas and Russell, 1997; Chai *et al.,* 1997]. It has also been proposed that liver microsomal retinol dehydrogenase (RoDH) may be more efficient than ADHs for retinol oxidation, since most of the retinol in liver cells is bound to cellular retinol-binding protein (CRBP), limiting access of retinol to ADHs, but facilitating access to RoDH through a reported specific interaction detected in vitro [Boerman and Napoli, 1995]. There has been no corroborative evidence, however, of such an interaction between CRBP and RoDH; moreover, it is unknown whether the active site of RoDH faces the cytoplasm where CRBP resides. Since most work has focused on liver, it is also unknown whether important retinoid target cells throughout the body contain sufficient CRBP to sequester most of the retinol. It is also unclear whether sequestration of retinol by CRBP can in fact hinder ADH to the point where this enzyme cannot contribute within a physiological context to the production of the very low nM quantities of retinoic acid seen in target tissues. In fact, since CRBP sequestration limits retinol utilization by both ADH4 [Kedishvili *et al.,* 1998] and RoDH [Napoli *et al.,* 1992], it is possible that CRBP functions as a sparing mechanism for this important vitamin by allowing it to be used only at nM levels. These problems indicate that additional methodologies such as the genetic studies reported in this paper are needed to advance our understanding of the enzymes involved in retinoic acid synthesis.

In summary, genetic studies provide evidence that *Adh4* mutant mice have a defect in retinol utilization under conditions of either vitamin A deficiency or excess. The lack of any obvious defect in *Adh4* mutant mice on a normal diet with moderate levels of vitamin A is most likely due to functional redundancy with *Adh1* or microsomal retinol dehydrogenase. Further genetic studies should answer this question.

ACKNOWLEDGMENTS

We thank R.J. Haselbeck for providing antibodies against mouse ADH1, ADH3, and ADH4; M. Wagner for the retinoic acid reporter cell line; D. Abbe and W. Charbono for help with retinol administration to mice; the Burnham Institute Mouse Genetics Facility; and H.L. Ang and I. Hoffmann for critical discussions. This work was supported by NIH grant AA09731 (to G.D.) and by an NCI Cancer Center grant to the Burnham Institute.

REFERENCES

Allali-Hassani A, Peralba JM, Martras S, Farrés J, Parés X. 1998. Retinoids, omega-hydroxyfatty acids and cytotoxic aldehydes as physiological substrates, and H_2-receptor antagonists as pharmacological inhibitors, of human class IV alcohol dehydrogenase. FEBS Lett 426:362–366.

Ang HL, Duester G. 1999. Stimulation of premature retinoic acid synthesis in Xenopus embryos following premature expression of aldehyde dehydrogenase ALDH1. Eur J Biochem (in press).

Ang HL, Deltour L, Hayamizu TF, Zgombic-Knight M, Duester G. 1996a. Retinoic acid synthesis in mouse embryos during gastrulation and craniofacial development linked to class IV alcohol dehydrogenase gene expression. J Biol Chem 271:9526–9534.

Ang HL, Deltour L, Zgombic-Knight M, Wagner MA, Duester G. 1996b. Expression patterns of class I and class IV alcohol dehydrogenase genes in developing epithelia suggest a role for alcohol dehydrogenase in local retinoic acid synthesis. Alcohol Clin Exp Res 20:1050–1064.

Biswas MG, Russell DW. 1997. Expression cloning and characterization of oxidative 17β- and 3α-hydroxysteroid dehydrogenases from rat and human prostate. J Biol Chem 272:15959–15966.

Bliss AF. 1951. The equilibrium between vitamin A alcohol and aldehyde in the presence of alcohol dehydrogenase. Arch Biochem 31:197–204.

Boerman MHEM, Napoli JL. 1995. Characterization of a microsomal retinol dehydrogenase: a short-chain alcohol dehydrogenase with integral and peripheral membrane forms that interacts with holo-CRBP (type I). Biochemistry 34:7027–7037.

Boleda MD, Saubi N, Farrés J, Parés X. 1993. Physiological substrates for rat alcohol dehydrogenase classes: aldehydes of lipid peroxidation, omega-hydroxyfatty acids, and retinoids. Arch Biochem Biophys 307:85–90.

Chai X, Boerman MHEM, Zhai Y, Napoli JL. 1995. Cloning of a cDNA for liver microsomal retinol dehydrogenase. A tissue-specific, short-chain alcohol dehydrogenase. J Biol Chem 270:3900–3904.

Chai XY, Zhai Y, Napoli JL. 1997. cDNA cloning and characterization of a *cis*-retinol/3α-hydroxysterol short-chain dehydrogenase. J Biol Chem 272:32411–32405.

Collins MD, Eckhoff C, Chahoud I, Bochert G, Nau H. 1992. 4-methylpyrazole partially ameliorated the teratogenicity of retinol and reduced the metabolic formation of all-*trans*-retinoic acid in the mouse. Arch Toxicol 66:652–659.

Connor MJ, Smit MH. 1987. Terminal-group oxidation of retinol by mouse epidermis: inhibition in vitro and in vivo. Biochem J 244:489–492.

Deltour L, Haselbeck RJ, Ang HL, Duester G. 1997. Localization of class I and class IV alcohol dehydrogenases in mouse testis and epididymis: potential retinol dehydrogenases for endogenous retinoic acid synthesis. Biol Reprod 56:102–109.

De Luca LM, Shores RL, Spangler EF, Wenk ML. 1989. Inhibition of initiator-promoter-induced skin tumorigenesis in female SENCAR mice fed a vitamin A-deficient diet and reappearance of tumors in mice fed a diet adequate in retinoid or β-carotene. Cancer Res 49:5400–5406.

Duester G. 1996. Involvement of alcohol dehydrogenase, short-chain dehydrogenase/reductase, aldehyde dehydrogenase, and cytochrome P450 in the control of retinoid signaling by activation of retinoic acid synthesis. Biochemistry 35:12221–12227.

Duester G, Farrés J, Felder MR, Holmes RS, Höög J-O, Parés X, Plapp BV, Yin S-J, Jörnvall H. 1999. Recommended nomenclature for the vertebrate alcohol dehydrogenase gene family. Biochem Pharmacol (in press).

Gough WH, VanOoteghem S, Sint T, Kedishvili NY. 1998. cDNA cloning and characterization of a new human microsomal NAD-dependent dehydrogenase that oxidizes all-*trans*-retinol and 3α-hydroxysteroids. J Biol Chem 273:19778–19785.

Han CL, Liao CS, Wu CW, Hwong CL, Lee AR, Yin SJ. 1998. Contribution to first-pass metabolism of ethanol and inhibition by ethanol for retinol oxidation in human alcohol dehydrogenase family—implications for etiology of fetal alcohol syndrome and alcohol-related diseases. Eur J Biochem 254:25–31.

Haselbeck RJ, Duester G. 1997. Regional restriction of alcohol/retinol dehydrogenases along the mouse gastrointestinal epithelium. Alcohol Clin Exp Res 21:1484–1490.

Haselbeck RJ, Duester G. 1998a. ADH1 and ADH4 alcohol/retinol dehydrogenases in the developing adrenal blastema provide evidence for embryonic retinoid endocrine function. Dev Dynam 213: 114–120.

Haselbeck RJ, Duester G. 1998b. ADH4-lacZ transgenic mouse reveals alcohol dehydrogenase localization in embryonic midbrain/hindbrain, otic vesicles, and mesencephalic, trigeminal, facial, and olfactory neural crest. Alcohol Clin Exp Res 22:1607–1613.

Haselbeck RJ, Ang HL, Deltour L, Duester G. 1997a. Retinoic acid and alcohol/retinol dehydrogenase in the mouse adrenal gland: a potential endocrine source of retinoic acid during development. Endocrinology 138:3035–3041.

Haselbeck RJ, Ang HL, Duester G. 1997b. Class IV alcohol/retinol dehydrogenase localization in epidermal basal layer: Potential site of retinoic acid synthesis during skin development. Dev Dynam 208:447–453.

Hogan B, Beddington R, Costantini F, Lacy E. 1994. Manipulating the mouse embryo. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Holmes RS. 1977. The genetics of α-hydroxyacid oxidase and alcohol dehydrogenase in the mouse: evidence for multiple gene loci and linkage between *Hao-2* and *Adh-3*. Genetics 87:709–716.

Joyner AL. 1993. Gene targeting: a practical approach. Oxford: IRL Press.

Jörnvall H, Danielsson O, Hjelmqvist L, Persson B, Shafqat J. 1995. The alcohol dehydrogenase system. Adv Exp Med Biol 372:281–294.

Kastner P, Chambon P, Leid M. 1994. Role of nuclear retinoic acid receptors in the regulation of gene expression. In: Blomhoff R, editor. Vitamin A in health and disease. New York: Marcel Dekker. p 189–238.

Kaufman MH. 1992. The atlas of mouse development. San Diego: Academic Press.

Kedishvili NY, Bosron WF, Stone CL, Hurley TD, Peggs CF, Thomasson HR, Popov KM, Carr LG, Edenberg HJ, Li T-K. 1995. Expression and kinetic characterization of recombinant human stomach alcohol dehydrogenase. Active-site amino acid sequence explains substrate specificity compared with liver isozymes. J Biol Chem 270:3625–3630.

Kedishvili NY, Gough WH, Davis WI, Parsons S, Li TK, Bosron WF. 1998. Effect of cellular retinol-binding protein on retinol oxidation by human class IV retinol alcohol dehydrogenase and inhibition by ethanol. Biochem Biophys Res Commun 249:191–196.

Labrecque J, Dumas F, Lacroix A, Bhat PV. 1995. A novel isoenzyme of aldehyde dehydrogenase specifically involved in the biosynthesis of 9-*cis* and all-*trans* retinoic acid. Biochem J 305:681–684.

Lee M-O, Manthey CL, Sladek NE. 1991. Identification of mouse liver aldehyde dehydrogenases that catalyze the oxidation of retinaldehyde to retinoic acid. Biochem Pharmacol 42:1279–1285.

Lohnes D, Mark M, Mendelsohn C, Dollé P, Dierich A, Gorry P, Gansmuller A, Chambon P. 1994. Function of the retinoic acid receptors (RARs) during development. (I) Craniofacial and skeletal abnormalities in RAR double mutants. Development 120:2723–2748.

Luo JM, Sucov HM, Bader JA, Evans RM, Giguère V. 1996. Compound mutants for retinoic acid receptor (RAR) β and RARα1 reveal developmental functions for multiple RARβ isoforms. Mech Dev 55:33–44.

Mangelsdorf DJ, Umesono K, Evans RM. 1994. The retinoid receptors. In: Sporn MB, Roberts AB, Goodman DS (eds): The retinoids: biology, chemistry, and medicine, 2nd ed. New York: Raven Press. p 319–349.

Mendelsohn C, Lohnes D, Décimo D, Lufkin T, LeMeur M, Chambon P, Mark M. 1994. Function of the retinoic acid receptors (RARs) during development. (II) Multiple abnormalities at various stages of organogenesis in RAR double mutants. Development 120:2749–2771.

Mertz JR, Shang EY, Piantedosi R, Wei SH, Wolgemuth DJ, Blaner WS. 1997. Identification and characterization of a stereospecific human enzyme that catalyzes 9-*cis*-retinol oxidation—a possible role in 9-*cis*-retinoic acid formulation. J Biol Chem 272:11744–11749.

Moreno A, Farrés J, Parés X, Jörnvall H, Persson B. 1996. Molecular modelling of human gastric alcohol dehydrogenase (class IV) and substrate docking: differences towards the classical liver enzyme (class I). FEBS Lett 395:99–102.

Morriss-Kay GM, Sokolova N. 1996. Embryonic development and pattern formation. FASEB J 10:961–968.

Napoli JL, Posch KC, Burns RD. 1992. Microsomal retinal synthesis: retinol vs. holo-CRBP as substrate and evaluation of NADP, NAD and NADPH as cofactors. Biochim Biophys Acta Protein Struct Mol Enzymol 1120:183–186.

Scott EW, Simon MC, Anastasi J, Singh H. 1994. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science 265:1573–1577.

Simon A, Hellman U, Wernstedt C, Eriksson U. 1995. The retinal pigment epithelial-specific 11-*cis*-retinol dehydrogenase belongs to the family of short chain alcohol dehydrogenases. J Biol Chem 270:1107–1112.

Smith SM, Levy NS, Hayes CE. 1987. Impaired immunity in vitamin A-deficient mice. J Nutr 117:857–865.

Wang XS, Penzes P, Napoli JL. 1996. Cloning of a cDNA encoding an aldehyde dehydrogenase and its expression in *Escherichia coli*—recognition of retinal as substrate. J Biol Chem 271:16288–16293.

Wellik DM, DeLuca H. 1995. Retinol in addition to retinoic acid is required for successful gestation in vitamin A-deficient rats. Biol Reprod 53:1392–1397.

Wellik DM, DeLuca HF. 1996. Metabolites of all-*trans*-retinol in day 10 conceptuses of vitamin A-deficient rats. Arch Biochem Biophys 330:355–362.

Wellik DM, Norback DH, DeLuca HF. 1997. Retinol is specifically required during midgestation for neonatal survival. Am J Physiol Endocrinol Metab 272:E25–E29.

White JA, Guo YD, Baetz K, Beckett-Jones B, Bonasoro J, Hsu KE, Dilworth FJ, Jones G, Petkovich M. 1996. Identification of the retinoic acid-inducible all-*trans*-retinoic acid 4-hydroxylase. J Biol Chem 271:29922–29927.

Xie PG, Parsons SH, Speckhard DC, Bosron WF, Hurley TD. 1997. X-ray structure of human class IV σσ alcohol dehydrogenase—Structural basis for substrate specificity. J Biol Chem 272:18558–18563.

Yang Z-N, Davis GJ, Hurley TD, Stone CL, Li T-K, Bosron WF. 1994. Catalytic efficiency of human alcohol dehydrogenases for retinol oxidation and retinal reduction. Alcohol Clin Exp Res 18:587–591.

Zachman RD, Olson JA. 1961. A comparison of retinene reductase and alcohol dehydrogenase of rat liver. J Biol Chem 236:2309–2313.

Zgombic-Knight M, Ang HL, Foglio MH, Duester G. 1995. Cloning of the mouse class IV alcohol dehydrogenase (retinol dehydrogenase) cDNA and tissue-specific expression patterns of the murine ADH gene family. J Biol Chem 270:10868–10877.

Zgombic-Knight M, Deltour L, Haselbeck RJ, Foglio MH, Duester G. 1997. Gene structure and promoter for *Adh3* encoding mouse class IV alcohol dehydrogenase (retinol dehydrogenase). Genomics 41:105–109.

Zhao D, McCaffery P, Ivins KJ, Neve RL, Hogan P, Chin WW, Dräger UC. 1996. Molecular identification of a major retinoic-acid-synthesizing enzyme, a retinaldehyde-specific dehydrogenase. Eur J Biochem 240:15–22.

Zile MH. 1998. Vitamin A and embryonic development: an overview. J Nutr 128 (suppl):455S–458S.