ADH1 and ADH4 Alcohol/Retinol Dehydrogenases in the Developing Adrenal Blastema Provide Evidence for Embryonic Retinoid Endocrine Function

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ABSTRACT **Studies on retinoid signaling indicate that much of the regulation of this pathway may involve enzymes that synthesize the active ligand retinoic acid. Alcohol dehydrogenases ADH1 (class I ADH) and ADH4 (class IV ADH) function as retinol dehydrogenases in the oxidation of retinol, a necessary step in the synthesis of retinoic acid from vitamin A. These enzymes as well as retinoic acid have previously been localized in the adult adrenal gland, thus providing evidence that this organ is an endocrine source of retinoic acid. Here, we have examined the involvement of ADH1 and ADH4 in embryonic adrenal function by using transgenic mouse technology and immunohistochemistry. Transgenic mice were generated that contain various portions of the mouse ADH4 promoter and 5′-flanking region fused to lacZ. Embryos harboring a construct containing 9.0 kb of 5′-flanking region displayed very high levels of lacZ expression in the developing adrenal blastemas at embryonic stage E11.5 during the initial phase of mouse adrenal gland development. The presence of endogenous ADH4 protein in stage E11.5 adrenal blastemas was demonstrated by immunohistochemistry, and this was the only site of ADH4 immunodetection in stage E11.5 embryos. Endogenous ADH1 protein was also detected by immunohistochemistry in stage E11.5 adrenal blastemas. ADH1 and ADH4 proteins were detectable at later stages of adrenal development, and both were localized to developing adrenal cortical cells by stage E14.5. The presence of both ADH1 and ADH4 retinol dehydrogenases during the earliest stages of adrenal gland development, combined with our earlier findings of high levels of retinoic acid in the embryonic adrenal gland, suggests that one of the earliest functions of ADH may be to provide an embryonic endocrine source of retinoic acid for growth and development.** ***Dev. Dyn. 1998;213:114–120.*** **© 1998 Wiley-Liss, Inc.**

Key words: adrenal; embryogenesis; mouse; alcohol dehydrogenase; retinol dehydrogenase; retinoic acid; transgenic mouse; class I ADH; class IV ADH

INTRODUCTION

Retinoic acid functions as a ligand controlling a nuclear receptor signaling pathway involved in vertebrate growth and development (Kastner et al., 1994; Mangelsdorf et al., 1994). The role of the retinoic acid receptor in vitamin A function has been firmly established by receptor knockout mice, which display the classic defects observed during vitamin A deficiency (Lohnes et al., 1994; Mendelsohn et al., 1994; Luo et al., 1996). An understanding of how retinoid metabolism is normally regulated to provide the ligand for this signaling pathway remains a major challenge in the study of vitamin A function. Mammalian alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) enzyme families participate in the metabolism of retinoids, particularly the oxidation of retinol to retinoic acid (as reviewed by Duester, 1996). Microsomal retinol dehydrogenase, a member of the short-chain dehydrogenase/reductase (SDR) enzyme family, has also been proposed to oxidize retinol in the presence or absence of cellular retinol-binding protein (Chai et al., 1995). Thus, further studies are needed to decipher the relative roles of the ADH and SDR families in retinol oxidation for retinoid signaling in vivo.

Mammalian ADHs are a family of cytosolic, zinc-dependent enzymes consisting of at least six distinct classes (Jörnvall et al., 1995; Duester et al., 1997). Amino acid sequence comparisons have revealed interclass sequence identities in the 57–69% range, indicating divergence from a common progenitor during early vertebrate evolution (Cederlund et al., 1991). ADH1 (class I ADH) and ADH4 (class IV ADH) are conserved among all mammalian lines examined and both catalyze retinol oxidation in vitro (Connor and Smit, 1987; Boleda et al., 1993; Yang et al., 1994; Kedishvili et al., 1995). Molecular modeling studies of human ADH1 and ADH4 based upon their known three-dimensional structures have shown that both enzymes possess large active sites that can easily accommodate retinol (Ke-

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dishvili et al., 1995; Whitmire et al., 1995; Moreno et al., 1996; Xie et al., 1997).

Mouse ADH1 and/or ADH4 have been localized in numerous retinoid-responsive epithelia, including the epidermis (Haselbeck et al., 1997b), testis (Deltour et al., 1997), genitourinary tract (Ang et al., 1996b), and digestive tract (Haselbeck and Duester, 1997), as well as in gastrula-stage embryos (Ang et al., 1996a; Ang and Duester, 1997), suggesting that these enzymes play a role in local retinoic acid synthesis. ADH1 and ADH4 are also localized in the adult adrenal gland, which has been demonstrated to contain significant levels of retinoic acid (Haselbeck et al., 1997a). These findings suggest that ADH may also participate in the production of retinoic acid as an endocrine hormone. Here, we have examined ADH1 and ADH4 during mouse embryogenesis and provide evidence that these enzymes may play a role in embryonic retinoid endocrine function in the developing adrenal gland.

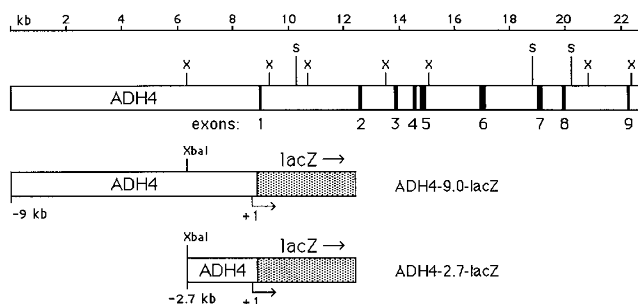

Fig. 1. ADH4 promoter-*lacZ* transgenes. A region of mouse genomic DNA containing the complete ADH4 gene is shown as previously described (Zgombic-Knight et al., 1997). Below the restriction map of the ADH4 gene (X, *Xba*I; S, *Sac*I) is shown two gene fusion constructs between the ADH4 promoter and lacZ, which differ in the amount of 5′-flanking DNA included. The transcription initiation site for the ADH4 gene is indicated by +1.

RESULTS

Production of Transgenic Mice Carrying ADH4 Promoter Fusions to lacZ

Previous characterization of the mouse ADH4 gene lead to the identification of all nine exons as well as 9.0 kb of 5′-flanking region containing a transcription initiation site upstream of exon 1 (Zgombic-Knight et al., 1997). To examine ADH4 expression during embryogenesis, fusion genes were constructed in which 9.0 kb and 2.7 kb of the ADH4 5′-flanking region plus promoter were fused to lacZ to produce ADH4–9.0-lacZ and ADH4–2.7-lacZ, respectively (Fig. 1). Southern blot analysis of mouse tail DNA was used to identify nine founders carrying the ADH4–9.0-lacZ transgene and seven founders carrying the ADH4–2.7-lacZ transgene. For ADH4–9.0-lacZ, we identified five founders that produced embryos in which transgene-derived β-galactosidase activity was observed in the developing adrenal gland based on in situ analysis of β-galactosidase activity (see data below). None of the ADH4–2.7-lacZ founders produced embryos that expressed the transgene in the adrenal gland (see data below). All subsequent studies described below were performed on founder 24 for ADH4–9.0-lacZ and founder 20 for ADH4–2.7-lacZ.

Expression of ADH4–9.0-lacZ in Embryonic Adrenal Gland of Transgenic Mice

Progeny of ADH4–9.0-lacZ and ADH4–2.7-lacZ founders were analyzed in situ for lacZ expression (β-galactosidase activity) during embryogenesis. ADH4–9.0-lacZ embryos at embryonic stage E15.5 exhibited easily detectable β-galactosidase activity in the adrenal gland localized primarily in the outer concentric zone of the developing cortex (Fig. 2A). On the other hand, ADH4–2.7-lacZ embryos exhibited no β-galactosidase activity in the developing adrenal gland at stage E15.5 (Fig. 2B) or at any earlier stages (data not shown). Examination of control nontransgenic embryos showed that there was no endogenous β-galactosidase activity in the adrenal gland at stage E15.5 (Fig. 2C) or at earlier stages (data not shown). Examination of embryos at E13.5, E14.5, and E16.5 indicated that adrenal expression was comparable to that shown at E15.5 (data not shown).

β-galactosidase activity was detected in the adrenal glands of ADH4–9.0-lacZ embryos at earlier stages examined, including stage E11.5 when the adrenal blastemas first can be identified (Fig. 2D) as well as stage E12.5 when the blastemas are larger in size (Fig. 2E). Tissue sections of E11.5 and E12.5 embryos stained for β-galactosidase activity revealed intense ADH4 transgene expression in the adrenal blastema cells (Fig. 2F,G). Examination of ADH4–9.0-lacZ embryos at stage E10.5 indicated no β-galactosidase activity in the trunk tissues, which later give rise to the adrenal blastema (data not shown).

ADH1 and ADH4 Immunohistochemical Detection During Adrenal Development

Strong expression of the ADH4 transgene in the developing adrenal blastemas prompted us to examine the localization of ADH protein as well. Immunohistochemical studies were performed on mouse embryos using specific affinity-purified antibodies for both mouse ADH1 and ADH4 (Haselbeck and Duester, 1997). ADH1 protein was easily detectable in the adrenal blastema at stage E11.5 (Fig. 3A), and ADH4 protein was weakly detectable at this stage (Fig. 3B) compared with a control in which the primary antibody was omitted (Fig. 3C). A higher magnification of the ADH1 and ADH4 immunohistochemical signals at stage E11.5 clearly shows that ADH1 immunodetection is strong and widespread throughout the adrenal blastema (Fig. 3D) and that ADH4 immunodetection is visible, although in fewer cells (Fig. 3E). Similar results were obtained for ADH1 and ADH4 at stage E12.5, and there was no

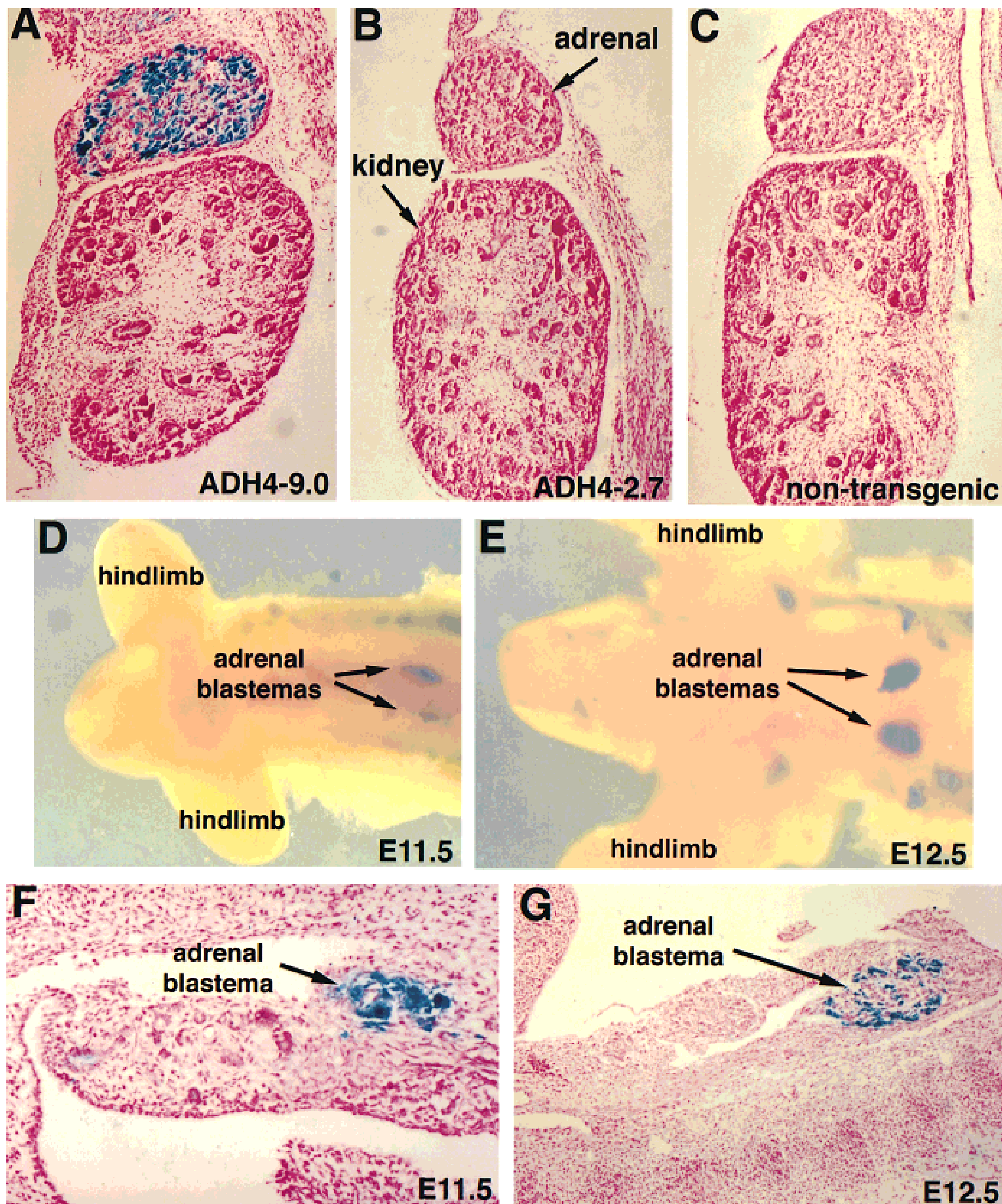

Fig. 2. Expression of ADH4–9.0 transgene in mouse embryonic adrenal gland. The adrenal gland and kidney of E15.5 embryos derived from transgenic line ADH4–9.0-lacZ (**A**), transgenic line ADH4–2.7-lacZ (**B**), and nontransgenic wild-type (**C**) are shown after whole-mount staining for β-galactosidase activity and sectioning. Whole-mount embryos of the transgenic line ADH4–9.0-lacZ were stained for β-galactosidase activity and opened with a scalpel to reveal internal staining of the adrenal blastemas at embryonic stage E11.5 (**D**) and stage E12.5 (**E**). Sections of whole-mount stained embryos show ADH4–9.0-lacZ expression in the adrenal blastema at stage E11.5 (**F**) and stage E12.5 (**G**). Original magnifications: ×25 in A,B,C, ×20 in D,E, ×50 in F,G.

immunodetection of ADH4 in any tissues other than the adrenal blastemas at E11.5 and E12.5, with ADH1 being detected at these stages only in the mesonephros as well as the adrenal blastemas (data not shown).

Immunohistochemical analysis of stage E14.5 adrenal glands showed that both ADH1 (Fig. 3F) and ADH4 (Fig. 3G) are easily detectable. Immunodetection of both ADH1 and ADH4 at E14.5 occurred primarily in the cells of the outer concentric zone where the cortex is forming, but significantly less cells had detectable ADH4 relative to ADH1 (Fig. 3F,G). Immunodetection of ADH1 and ADH4 at stages E15.5–E16.5 was essen-

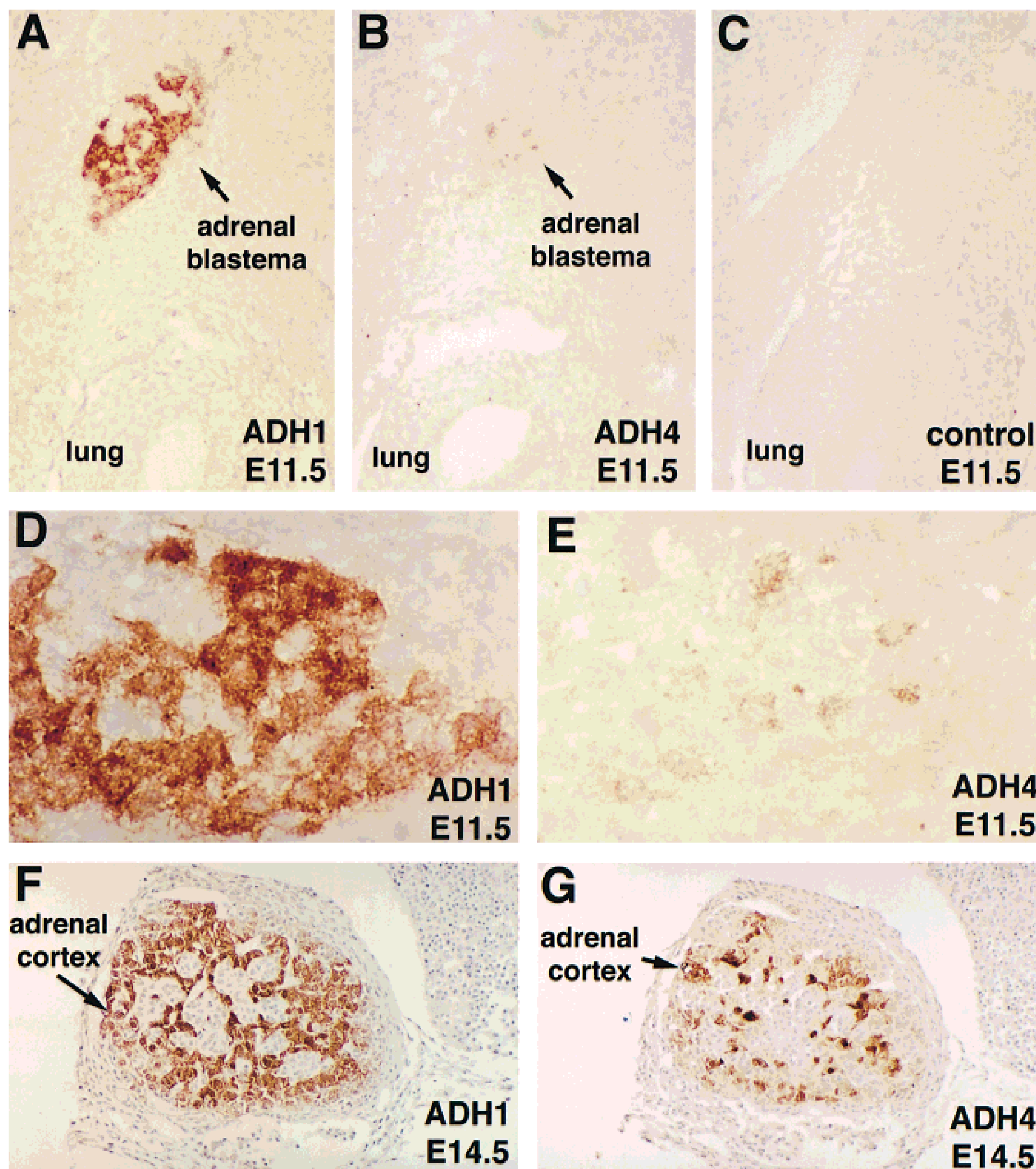

Fig. 3. Immunohistochemical localization of ADH1 and ADH4 during adrenal development. Transverse sections through the adrenal blastema of embryonic stage E11.5 mouse embryos were subjected to immunodetection with antibodies specific for ADH1 (**A**) or ADH4 (**B**) or treated as a control by omission of the primary antibody (**C**). Also shown is a higher magnification of the ADH immunodetection signals observed above in the E11.5 adrenal blastema for ADH1 (**D**) and ADH4 (**E**). Sections through a stage E14.5 adrenal gland were subjected to immunodetection with antibodies for ADH1 (**F**) and ADH4 (**G**), which localize in cells of the developing adrenal cortex. Original magnifications: ×50 in A,B,C,F,G, ×200 in D,E.

tially in the same pattern as that described for stage E14.5 (data not shown).

DISCUSSION

The studies reported here demonstrate that ADH1 and ADH4 are both expressed in the mouse embryonic adrenal gland at the earliest stages of its development. Immunohistochemical observations show that both ADH1 and ADH4 proteins are present in the adrenal blastema at stage E11.5 and, thereafter, in the developing adrenal cortex. Furthermore, the ADH4–9.0-lacZ transgenic mouse studies provide vivid evidence that the ADH4 gene is transcribed in the adrenal blastema at stage E11.5 and beyond. During stages E11.5–E12.5, immunohistochemical analyses indicate that ADH1 and ADH4 expression is quite limited, with ADH4 immunodetection observed only in the adrenal blastema and with ADH1 immunodetection limited to the adrenal blastema plus the mesonephros. Tissue-specific expression of ADH1 and ADH4 in the adrenal blastema

suggests that these enzymes play an important role in early embryonic endocrine function.

In the adult adrenal gland, both ADH1 and ADH4 immunodetection have previously been observed to be restricted to the cortical tissue, with no detectable signal in the medulla (Haselbeck et al., 1997a). In the embryonic adrenal gland, the studies presented here demonstrate that cells exhibiting ADH1 and/or ADH4 immunodetection appear to lie primarily in an outer concentric zone of the developing adrenal gland where the cortical cells are assembling to eventually form a distinct cortex surrounding the medulla. Previous studies have shown that ADH4 is present in significantly less adult adrenal cortical cells than ADH1; expression is radially variegated with only about 50% of the cortical cells having detectable ADH4 protein occurring as radial spokes around the medulla (Haselbeck et al., 1997a). The significance of this ADH4 expression pattern is unknown, but it is interesting that the ADH1 and ADH4 immunodetection studies described here show that the early embryonic adrenal gland appears to have already acquired this differential expression because clearly less cells express ADH4 relative to ADH1 between stages E11.5 and E14.5.

Previous analyses of ADH1 and ADH4 expression by in situ hybridization have shown that mRNAs for both enzymes are detectable in the adrenal gland during embryogenesis at stages E14.5–E16.5 as well as in the adult adrenal cortex (Ang et al., 1996b). The transgenic mouse studies reported here provide evidence that the ADH4 gene is transcribed in the embryonic adrenal gland at stages E14.5–E16.5 and have also led to the detection of transcription in the developing adrenal blastema during the earliest phase of its differentiation at stages E11.5–E12.5. We observed that the intensity of lacZ detection in the adrenal blastemas of ADH4–9.0-lacZ embryos at stage E11.5 was much higher than the intensity of ADH4 immunodetection at the same stage. This finding may be attributable to the higher signal-to-noise ratio of the lacZ stain relative to the peroxidase stain used for immunodetection. Alternatively, ADH4 gene transcription (measured by the lacZ assay) may be more abundant than ADH4 mRNA translation, resulting in a higher signal with the lacZ transgene than with immunodetection. These transgenic studies have also provided some localization of the regulatory elements controlling adrenal ADH4 transcription. Although sequences downstream of the promoter were not examined, we did examine an extensive region of 5′-flanking DNA including 9.0 kb of upstream sequences. Our observation of adrenal-specific expression for the ADH4–9.0-lacZ transgene, but not the ADH4–2.7-lacZ transgene, indicates that DNA elements controlling adrenal expression lie between 2.7- and 9.0-kb upstream of the ADH4 transcription initiation site. Previous studies on the ADH1 promoter in transgenic mice have provided evidence that the regulatory elements controlling adult adrenal gland transcription lie within the first 2.5-kb upstream of the transcription initiation site of this ADH gene (Xie et al., 1996). Thus, there is evidence that the genes for both retinol-active forms of mouse ADH (ADH1 and ADH4) contain transcriptional regulatory elements for adrenal expression.

Studies on the mammalian ADH enzyme family have provided evidence that several ADHs including ADH1 and ADH4 can catalyze retinol oxidation needed for the synthesis of retinoic acid (Connor and Smit, 1987; Boleda et al., 1993; Yang et al., 1994; Kedishvili et al., 1995). The expression patterns of ADH1 and ADH4 in retinoid-responsive tissues of the mouse further support this contention (Ang et al., 1996a, 1996b; Haselbeck et al., 1997b; Deltour et al., 1997; Haselbeck and Duester, 1997; Ang and Duester, 1997). In particular, the discovery that ADH1 and ADH4 are expressed in the mouse adult adrenal cortex as well as the detection of significant amounts of retinoic acid in the adrenal glands of adult and E16.5 embryonic mice have led to the hypothesis that these ADHs function to produce retinoic acid in the adrenal gland for endocrine distribution (Haselbeck et al., 1997a). These earlier findings combined with those presented here suggest that ADH1 and ADH4 may function as retinoid metabolic enzymes at the earliest stage of adrenal development. This may lead to the production of retinoic acid for embryonic endocrine distribution starting at stage E11.5 when the adrenal blastemas develop.

A special role for retinol in survival of rat embryos has been observed during the stage when the adrenal blastema develops. In particular, vitamin A-depleted female rats maintained on a diet containing a low dose of retinoic acid can become pregnant; however, the embryos are resorbed by stage E15.5 unless a dose of retinol is administered by stage E10.5–E11.5 (Wellik and DeLuca, 1995; Wellik et al., 1997). These findings indicate that retinoic acid can be distributed in a systemic fashion (i.e., analogous to endocrine distribution) from the diet of the mother to the embryos to support early development, but that this is not sufficient for development after stage E11.5. Because retinol is generally accepted to be a prohormone unable to carry out signaling events on its own, the reason for its requirement after stage E11.5 is presumably to function as a substrate that is metabolized to active forms such as retinal or retinoic acid or some other form that may be important for maintenance of the entire embryo during late gestation (Wellik and DeLuca, 1996). Our observation that retinol-active ADHs are specifically localized in the adrenal blastemas at stage E11.5 during the time when retinol must be present for overall embryonic survival suggests that the retinol requirement may involve conversion of retinol to retinal in the adrenal blastema by ADH. Either retinal, retinoic acid, or an unknown metabolite may then be distributed in an endocrine fashion throughout the embryo to reach retinoid-dependent tissues and allow embryonic survival.

EXPERIMENTAL PROCEDURES

Construction of ADH4-lacZ Transgenes

A 3.8-kb fragment containing the *Escherichia coli* lacZ gene encoding β-galactosidase followed by an SV40 polyadenylation signal was excised from pCH110 (Pharmacia, Uppsala, Sweden) with *Hin*dIII and *Bam*HI, then ligated into pIC19R (Marsh et al., 1984) to form pIC19R-lacZ as previously described (Zgombic-Knight et al., 1994). A 2.7-kb fragment containing the mouse ADH4 promoter was derived by polymerase chain reaction (PCR) from a 3.0-kb genomic *Xba*I fragment containing the 5′ end of ADH4 cloned in pBluescript II KS (Zgombic-Knight et al., 1997). The PCR primers consisted of the KS primer (upstream of the ADH4 promoter in the plasmid multiple cloning site) and a downstream primer located in the 5′-untranslated region of ADH4 (5′-CATCGACTCGAGATCCTGCCTCTCCTGGGTG-3′). This 2.7-kb promoter fragment was digested with *Bam*HI and *Xho*I and ligated upstream of lacZ between the *Bgl*II and *Xho*I sites of pIC19R-lacZ to form ADH4–2.7-lacZ, which generated a transgene containing the ADH4 promoter and 2.7 kb of 5′-flanking DNA upstream of lacZ. In this gene fusion the ATG translation start codon for lacZ is the first such codon downstream of the ADH4 transcription initiation site. ADH4–9.0-lacZ was constructed by ligating a 6.3-kb genomic *Xba*I fragment containing only 5′-flanking DNA of ADH4 (Zgombic-Knight et al., 1997) into the *Xba*I site of the plasmid ADH4–2.7-lacZ to generate a transgene containing the ADH4 promoter and 9.0 kb of 5′-flanking DNA upstream of lacZ.

Production of Transgenic Mice

A 6.5-kb *Spe*I-*Bam*HI DNA fragment containing ADH4–2.7-lacZ and a 12.8-kb *Spe*I-*Bam*HI DNA fragment containing ADH4–9.0-lacZ were separated from their respective plasmid vector DNAs by agarose gel electrophoresis, then injected (2 ng ml^{-1}) into the male pronucleus of fertilized mouse eggs (FVB female × C57BL6 male), which were then transferred to pseudopregnant FVB females as described (Hogan et al., 1986). Several founders were identified that carried each transgene based upon Southern blot analysis of tail DNA using a lacZ DNA probe (Hogan et al., 1986). Founders that passed the transgene on to offspring based upon by Southern blot analysis of tail DNA were used for further studies.

Staging of Embryos

Embryos were staged by vaginal plug appearance with noon on the day of plug detection being considered E0.5. Further staging of mouse embryos was according to Kaufman (1992).

Transgenic Mouse Embryo Analysis

Embryos at E10.5-E15.5 were analyzed. After removal of the embryo from extraembryonic tissues, staining for β-galactosidase activity was performed as previously described (Zgombic-Knight et al., 1994). Briefly, embryos were fixed in a solution containing 2% formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40 in phosphate buffered saline (PBS), for 60 min on ice. The samples were rinsed with PBS and stained at 37°C for 16 hr in a solution containing 1 mg ml^{-1} X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside), 5 mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ in PBS. Samples were post-fixed in 4% paraformaldehyde and photographed. In some cases, specimens were cut open with a scalpel before photography to more easily reveal internal staining. In all cases, nontransgenic embryos of the same stage were analyzed for endogenous β-galactosidase activity using the identical staining conditions.

After whole-mount β-galactosidase detection, samples were embedded in paraffin and sectioned at 7 mm using standard methodology (Kaufman, 1992). Sections were counterstained with eosin before mounting and bright-field microscopic observation.

Immunohistochemistry

Antibodies used in this study were affinity-purified from rabbit polyclonal antisera raised against mouse ADH1 and ADH4 expressed as glutathione-*S*-transferase fusion proteins in *E. coli* as previously described (Haselbeck and Duester, 1997). Western blot analysis was used to verify that no cross-reactivity existed between the ADH1 and ADH4 antibodies as detailed previously (Haselbeck and Duester, 1997). Whole-embryo paraffin sections were subjected to immunohistochemical detection using the Vectastain avidin-biotin-horseradish peroxidase ABC kit (Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine for color detection as previously described (Haselbeck et al., 1997a). In control experiments the primary antibody was omitted.

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