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Distinct Functions for *Aldh1* and *Raldh2* in the Control of Ligand Production for Embryonic Retinoid Signaling Pathways

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Abstract

During vertebrate embryogenesis retinoic acid (RA) synthesis must be spatiotemporally regulated in order to appropriately stimulate various retinoid signaling pathways. Various forms of mammalian aldehyde dehydrogenase (ALDH) have been shown to oxidize the vitamin A precursor retinal to RA in vitro. Here we show that injection of *Xenopus* embryos with mRNAs for either mouse *Aldh1* or mouse *Raldh2* stimulates RA synthesis at low and high levels, respectively, while injection of human *ALDH3* mRNA is unable to stimulate any detectable level of RA synthesis. This provides evidence that some members of the ALDH gene family can indeed perform RA synthesis in vivo. Whole-mount immunohistochemical analyses of mouse embryos indicate that ALDH1 and RALDH2 proteins are localized in distinct tissues. RALDH2 is detected at E7.5–E10.5 primarily in trunk tissue (paraxial mesoderm, somites, pericardium, midgut, mesonephros) plus transiently from E8.5–E9.5 in the ventral optic vesicle and surrounding frontonasal region. ALDH1 is first detected at E9.0–E10.5 primarily in cranial tissues (ventral mesencephalon, dorsal retina, thymic primordia, otic vesicles) and in the mesonephros. As previous findings indicate that embryonic RA is more abundant in trunk rather than cranial tissues, our findings suggest that *Raldh2* and *Aldh1* control distinct retinoid signaling pathways by stimulating high and low RA biosynthetic activities, respectively, in various trunk and cranial tissues.

Keywords

retinoic acid; aldehyde dehydrogenase; *Aldh1*; *Raldh2*; retinoid signaling

INTRODUCTION

Vitamin A (retinol) plays a role in the regulation of vertebrate embryonic development through its active metabolite retinoic acid (RA) [reviewed by Hofmann and Eichele, 1994; Gudas *et al.*, 1994; Maden, 1994]. RA functions as a ligand for several nuclear retinoic acid receptors (RARs) which function as ligand-dependent transcription factors in conjunction with retinoid-X receptors (RXRs), with which they form heterodimers [reviewed by Kastner *et al.*, 1994; Mangelsdorf *et al.*, 1994]. Essential roles for these receptors in vitamin A

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function have been confirmed in mice carrying targeted mutations of RARs and RXRs [Lohnes *et al.*, 1994; Mendelsohn *et al.*, 1994; Luo *et al.*, 1996; Mascrez *et al.*, 1998]. A major challenge remaining in the study of retinoid signaling is the elucidation of how production of the ligand is spatiotemporally regulated in various tissues during development to provide the correct amount of RA for each discrete receptor-mediated signaling event.

The major biosynthetic pathway of RA from retinol involves two sequential steps catalyzed by several candidate enzymes [reviewed by Duester, 1996; Napoli, 1996]. First, reversible dehydrogenation of retinol into retinal is catalyzed by either cytosolic retinol dehydrogenases, which are members of the alcohol dehydrogenase (ADH) family, or by microsomal retinol dehydrogenases, which are members of the short-chain dehydrogenase/reductase family (SDR). Mice carrying a targeted *Adh4* null mutation have defects in retinol utilization during vitamin A deficiency [Deltour *et al.*, 1996b] and *Adh1*−/− mice display significant reductions in the ability to metabolize a dose of retinol to RA [Deltour *et al.*, 1999a]. Thus, at least two forms of ADH are involved in metabolizing retinol to retinal in vivo.

In the second step of the metabolic pathway, retinal is irreversibly oxidized to RA by cytosolic retinal dehydrogenases, which are members of the aldehyde dehydrogenase (ALDH) family. Examination of the second step of RA synthesis in vitro originally led to the identification of calf liver ALDH as an enzyme able to oxidize retinal to RA [Futterman, 1962]. Subsequent studies revealed an extensive family of mammalian ALDHs, with the original form active for RA synthesis corresponding to class I ALDH (ALDH1) in the human [Dockham *et al.*, 1992; Yoshida *et al.*, 1992], as well as its mouse ALDH1 homolog previously known as AHD2 [Lee *et al.*, 1991; McCaffery *et al.*, 1992], and its rat ALDH1 homolog also known as RALDH or RalDH-I [Labrecque *et al.*, 1995; Penzes *et al.*, 1997; Kathmann and Lipsky, 1997]. Two additional classes of ALDH, i.e., mitochondrial ALDH2 and cytosolic ALDH3, have been shown to have no RA synthetic activity in vitro [Yoshida *et al.*, 1992]. The discovery that mouse ALDH1 is localized during embryogenesis in the dorsal retina (a retinoid-target tissue) provided impetus to examine this enzyme as a potential embryonic RA synthetic enzyme [McCaffery *et al.*, 1991]. Further studies in the mouse culminated in the discovery of an additional RA synthetic enzyme identified as a distinct class of ALDH sharing approximately 70% amino acid sequence identity with ALDH1; this form has been named RALDH2 in mouse [Zhao *et al.*, 1996], human [Ono *et al.*, 1998], and chick [Sockanathan and Jessell, 1998], and RalDH-II in the rat [Wang *et al.*, 1996].

During early mouse embryogenesis, there exists a posterior preference for initial RA synthesis [Hogan *et al.*, 1992]. This may help establish the anteroposterior axis during early development due to regulation of members of the Hox gene family, which are RA-inducible and differentially expressed along the anteroposterior axis with expression only in posterior tissues (trunk and posterior hindbrain) [Shimeld, 1996]. In addition to anteroposterior patterning, RA does play a role in cranial development, as demonstrated by embryonic vitamin A-deficiency studies [Maden *et al.*, 1996; Dickman *et al.*, 1997] as well as gene knockouts of RARs [Lohnes *et al.*, 1994] and *Raldh2* [Niederreither *et al.*, 1999]. Thus, RA must exist in cranial locations as well as trunk locations. Localization of RA in mouse

embryos has been determined using either a transgenic RA reporter mouse line [Rossant *et al.*, 1991] or a whole-embryo explant RA bioassay [Ang *et al.*, 1996a] which employs an RA reporter cell line that was originally used to identify RA in the neural tube [Wagner *et al.*, 1992]. In both assays, RA is initially detected at E7.5, limited to posterior tissues, and then from E8.5–E10.5 RA is still abundant in the trunk, but it is now also detected in the optic vesicles and surrounding frontonasal region. *Raldh2* mRNA transcripts are initially expressed in the posterior mesoderm of mouse embryos at E7.5, with additional expression in the heart at E8.25, transient expression in the optic vesicles at E8.5, and a continued high level of expression in the trunk from stages E8.5–E10.5 and in the spinal cord by E12.5 [Zhao *et al.*, 1996; Niederreither *et al.*, 1997; Moss *et al.*, 1998]. *Raldh2* −/− null mutant mice die at midgestation, displaying no detectable RA in the trunk and frontonasal region at late E8.5, but with some RA still detectable in the optic vesicles [Niederreither *et al.*, 1999]. Thus, *Raldh2* appears to be responsible for essentially all of the RA synthesis occurring in the trunk and frontonasal regions by late E8.5, but not for all RA synthesis occurring in the optic vesicles. A role for mouse *Aldh1* in optic vesicle RA synthesis is suggested based on its expression in the dorsal retina at E9.5 [McCaffery *et al.*, 1991]; however, a more complete analysis of *Aldh1* is needed to determine the full extent of its role in RA synthesis.

A direct demonstration of the abilities of *Aldh1* and *Raldh2* to catalyze embryonic RA synthesis by overexpression in an in vivo setting has not been previously reported. Also, the localization of both ALDH1 and RALDH2 proteins during embryogenesis has not been adequately addressed in order to identify tissues where the enzymes actually exist and RA synthesis can thus be expected to occur. Here, we examined mouse *Aldh1* and *Raldh2* for their ability to function in RA synthesis in vivo by expression in *Xenopus* embryos. Our results provide firm evidence that both genes stimulate RA synthesis when expressed in *Xenopus*, and these findings provide insight as to their relative abilities. In addition, we used specific antibodies directed against ALDH1 and RALDH2 to demonstrate distinct patterns of protein localization for these two enzymes in mouse cranial and trunk embryonic tissues, thus defining distinct domains where *Aldh1* and *Raldh2* may influence retinoid signaling during development.

MATERIALS AND METHODS

ALDH cDNAs

A full-length cDNA for mouse *Aldh1* (originally known as *Ahd2*) has been previously described [Hsu *et al.*, 1999]. A full-length cDNA for human *ALDH3* has also been described [Hsu *et al.*, 1992].

A full-length cDNA for mouse *Raldh2* was obtained as follows. Total RNA was isolated from adult mouse testis by the acid guanidinium thiocyanate-phenolchloroform method [Chomczynski and Sacchi, 1987]. cDNA cloning was performed by reverse transcription coupled by polymerase chain reaction (PCR). First strand cDNA synthesis was performed on 5 μg of total RNA using oligo-dT as a primer and AMV reverse transcriptase, as described in the cDNA synthesis kit supplied by Amersham Life Science Inc. (Arlington Hts., IL). The single-stranded cDNA product was subjected to PCR using standard methodology [Ausubel *et al.*, 1989] with oligonucleotide primers corresponding to the 5′

and 3′ regions of the mouse *Raldh2* sequence encompassing the start and stop codons, respectively [Zhao *et al.*, 1996]. PCR products of approximately 1,600 bp were cloned into Bluescript IIKS (Stratagene Cloning Systems, La Jolla, CA) and a clone containing the complete open reading frame of mouse *Raldh2* was verified by DNA sequence analysis.

RNA Transcription and Translation

Full-length cDNAs for mouse *Aldh1*, mouse *Raldh2*, and human *ALDH3* were subcloned into plasmid pSP65 (Promega, Madison, WI) in the sense direction for in vitro transcription from the SP6 promoter. Plasmids were linearized and subjected to in vitro transcription with SP6 RNA polymerase, 5′-capping with 7-methylguanosine, followed by 3′-polyadenylation with *Escherichia coli* poly(A) polymerase to produce full-length mRNAs [Vize *et al.*, 1991]. The mRNAs generated in this fashion were brought to a concentration of 0.2 μg/μl in water, and 0.4 μg was used for in vitro translation in a rabbit reticulocyte lysate (Stratagene, CA) to monitor the ability of the mRNAs to produce full-length proteins.

Frog Embryo Microinjection and RA Detection

Xenopus laevis embryos were produced by artificial fertilization and staged as described previously by Nieuwkoop and Faber [1994]. Embryos were placed in 1× MMR, 3% Ficoll-400 at room temperature for microinjection of mRNA as described previously [Kay, 1991]. Various amounts of mRNA (4.6–23.0 nl at a concentration of either 0.2 ng/ml in water) was injected into the vegetal pole of embryos at the 2–4 cell stages using a Nanoject microinjection apparatus (Drummond Scientific, Broomall, PA) attached to the micropipet prefilled with light mineral oil. Four hours after injection, embryos were transferred to 0.1× MMR and incubated to stage 8 (blastula).

RA was detected using a bioassay that employs the RA reporter cell line F9-RARE-lacZ [Wagner *et al.*, 1992]. This cell line was derived from stable transfection of mouse F9 cells with a transgene containing an RA response element driving expression of *lacZ*. F9-RARE-lacZ detects the presence of RA in cultured embryo explants by detecting diffusion of this lipid-soluble molecule from the embryo to the surrounding reporter cells. Bioassay of *Xenopus* RA was performed as previously described for mouse embryos [Ang *et al.*, 1996a]. Briefly, F9-RARE-lacZ cells were grown until 80–90% confluent, at which time stage 8 *Xenopus* embryos were placed on top of the reporter cell monolayer, incubated for 18 h, then fixed in 1% glutaraldehyde and assayed for β-galactosidase activity produced by *lacZ* expression. *Xenopus* embryos placed upon the reporter cells and incubated under standard mammalian cell culture conditions did not develop further, but remained intact for the duration of the assay.

Production of Antibodies and Western Blot Analysis

Mouse ALDH1 and RALDH2 proteins were expressed in the *E. coli* strain BL-21 as N-terminal fusions to *Schistosoma* glutathione-S-transferase (GST) using pGEX expression vectors (Pharmacia Biotech, Uppsala, Sweden). A 1.6 kb full-length cDNA for mouse *Aldh1* [Hsu *et al.*, 1999] was cloned downstream of GST in the EcoRI site of pGEX-4T-2. Likewise, a 1.6 kb full length cDNA for mouse *Raldh2* (described above) was cloned into the EcoRI site of pGEX-4T-3. Both constructions resulted in fusion proteins with

approximate molecular weights of 84,000 (GST ~29 kDa plus ALDH ~55 kDa) after induction of transformed BL-21 cultures with isopropyl-β-D-thiogalactopyranoside (IPTG) at 0.1%. Inclusion body preparations of ALDH1-GST and RALDH2-GST fusion proteins prepared as described [Domingo *et al.*, 1992] were solubilized in Laemmli sample buffer and resolved on preparative SDS-PAGE gels using standard techniques [Sambrook *et al.*, 1989]. After staining the gel with 0.05% coomassie brilliant blue in water for 1 h, the 84 kDa fusion proteins were excised and divided into 200 μg aliquots. Each of these were mascerated in Freund's adjuvant and used to perform four monthly injections into rabbits using standard techniques [Harlow and Lane, 1988]. Antibodies were affinity-purified from antisera using 100 μg aliquots of the specific ALDH-GST fusion protein electroblotted to PVDF membrane, and were quantitated as previously described [Haselbeck and Duester, 1997].

Western blot analysis was performed on homogenates of adult mouse tissues (FVB/N) as previously described [Haselbeck and Duester, 1997].Western blots were subjected to immunochemical detection using the ECL chemiluminescence detection kit (Amersham). ALDH1 and RALDH2 primary antibodies were used at a concentration of 0.02 μg/ml. Electroblotted membranes were stripped and reanalyzed using each affinity-purified antibody.

Immunohistochemical Analysis of Mouse Embryos

Mouse embryos (FVB/N) were staged by vaginal plug appearance with noon on the day of plug detection being considered embryonic day 0.5 (E0.5). Further staging of mouse embryos was according to Kaufman [1992]. For all immunohistochemical analyses, ALDH1 and RALDH2 primary antibodies were used at a concentration of 1.0 μg/ml.

Whole-mount immunohistochemistry was performed on mouse embryos from stages E7.5–E10.5 using the above affinity-purified ALDH antibodies as described previously [Hogan *et al.*, 1994]. Color detection was performed by incubation with diaminobenzidine/nickel chloride for 30 min at room temperature followed by addition of hydrogen peroxide and further incubation for up to 60 min. Prior to photography, embryos were cleared in benzyl alcohol:benzyl benzoate (1:2) for 10 min.

Additional immunohistochemical studies were performed on mouse embryos embedded in paraffin and sectioned at 6 μm using standard methodology [Kaufman, 1992]. ALDH localization was performed by incubation for 30 min with purified antibody serially diluted in normal blocking solution, followed by detection using the Vectastain Elite avidin-biotin-horseradish peroxidase ABC kit (Vector Laboratories, Burlingame, CA). Incubation with diaminobenzidine for color detection was performed for 3 min essentially as recommended by the Vectastain kit, followed by dilution in tap water to stop the reaction. Slides were lightly counterstained with hematoxylin prior to mounting and observation by bright-field microscopy.

RESULTS

Aldh1 and *Raldh2* mRNAs Stimulate RA Synthesis in *Xenopus* Embryos

In order to explore the ability of certain ALDH genes to regulate RA synthesis in vivo, we injected mouse *Aldh1*, mouse *Raldh2*, or human *ALDH3* mRNAs into *Xenopus* embryos at the 2–4 cell stage, incubated the embryos to the blastula stage, then monitored RA levels in individual embryos using a *lacZ*-linked bioassay. Whereas RA was not detectable in uninjected embryos (0 out of 45 examined) nor in embryos injected with *ALDH3* mRNA (0 out of 47 embryos injected), a significant amount of RA was detectable in embryos injected with either *Raldh2* mRNA (36 out of 36 embryos injected) or *Aldh1* mRNA (47 out of 61 embryos injected) (Fig. 1). *Raldh2* mRNA was more efficient in this regard than *Aldh1* mRNA, as evidenced by a much larger area of *lacZ* detection around the embryo, but both of these mRNAs were clearly distinguishable from *ALDH3* mRNA, which completely lacked the ability to stimulate detectable RA synthesis. The mRNAs prepared from *Raldh2*, *Aldh1*, and *ALDH3* were each able to be translated into a polypeptide of approximately 55 kDa, corresponding to the expected size of ALDH subunits (Fig. 2).

Distribution of ALDH1 and RALDH2 Proteins in Adult Mouse Tissues

Since forced expression of mouse *Aldh1* and *Raldh2* genes in *Xenopus* embryos resulted in RA synthesis, we generated antibodies against both of these enzymes to examine their distribution patterns in the mouse. Polyclonal antibodies raised against full-length proteins for mouse ALDH1 and RALDH2 were used to probe adult mouse tissue extracts by Western blot analysis. Both antibodies detected proteins of 55 kDa, the expected size of an ALDH subunit, but the distribution of each protein was quite distinct. ALDH1 immunodetection was observed in extracts of the whole eye, lung, esophagus (weak), stomach, liver, small intestine, large intestine, adrenal, ovary, uterus, testis, and epididymis, with no detection observed in extracts of the whole brain, thymus, heart, kidney, or skin (Fig. 3). On the other hand, after examination of all the above tissues with RALDH2 antibodies, immunodetection was observed in only a very limited set of tissues, including the ovary (weak), uterus, and testis (Fig. 3). Based on our observed widespread appearance of ALDH1 in numerous adult organs containing retinoid-dependent epithelia, but a much more restricted localization of RALDH2 to a few reproductive organs,ALDH1 is likely to play a much wider role than RALDH2 in RA synthesis for adult tissues.

Our distribution of ALDH1 protein agrees with the expression pattern of *Aldh1* mRNA, which has previously been observed in mouse liver, intestine, lung, and testis, but not in kidney, brain or heart [López-Fernández and Del Mazo, 1997; Hsu *et al.*, 1999]. The specificity of the ALDH1 antibody is also shown by the presence of a strong signal in the lung, liver, and testis, but a complete absence of a signal in the kidney (Fig. 3), results which match previous findings on the distribution of mouse ALDH1 enzyme activity in adult organs [Rout and Holmes, 1985]. The specificity of the RALDH2 antibody is shown by our detection of a protein signal in the testis but not liver (Fig. 3), thus matching previous studies on *Raldh2* mRNA distribution in these adult tissues [Zhao *et al.*, 1996]. Since the adult mouse liver contains abundant ALDH1 enzyme activity [Rout and Holmes, 1985] and *Aldh1* mRNA [López-Fernández and Del Mazo, 1997], the absence of a signal in the liver

indicates that the RALDH2 antibody does not detect ALDH1. Also, the ALDH1 and RALDH2 antibodies do not recognize another related cytosolic enzyme, ALDH-PB, as evidenced by the lack of a signal in the adult kidney (Fig. 3), which has previously been shown to contain abundant ALDH-PB [Hsu *et al.*, 1999]. As these results demonstrate that the ALDH1 and RALDH2 antibodies have a high degree of specificity and do not display significant cross-reaction with each other or with ALDH-PB, they are thus suitable for studies on embryonic protein localization.

Distinct Cranial and Trunk Localization of ALDH1 and RALDH2 Proteins During Early Mouse Embryogenesis

The overall results of our whole-mount immunohistochemical analyses of ALDH1 and RALDH2 during stages E7.5–E10.5 of mouse embryogenesis are summarized in Table 1. Embryos older than E10.5 were not examined in detail, but selected organs were examined.

ALDH1 protein was not detected at E7.5 (data not shown) or E8.5 (Fig. 4A), but was first detected at E9.0 in a cranial location, particularly in the ventral flexure of the mesencephalon (Fig. 4B). By E9.5, ALDH1 protein was detected in several cranial derivatives, including the ventral mesencephalon, the dorsal retina, and the thymic primordia (Fig. 4C). At E10.5, ALDH1 protein was observed cranially in the ventral mesencephalon, dorsal retina, thymic primordia, and otic vesicles, and in the one trunk tissue, the mesonephric ducts (Fig. 4D). Interestingly, along the anteroposterior axis of the mesonephros, ALDH1 localization was much higher in those regions just posterior to the forelimb and hindlimb buds (Fig. 4D).

RALDH2 localization was for the most part nonoverlapping with that of ALDH1, occurring primarily in trunk tissues. RALDH2 protein was detected at E7.5–E8.0 in paraxial mesoderm up to the base of the headfolds (data not shown). At E8.5–E9.5, RALDH2 protein was detected in the developing somites, paraxial mesoderm, coelomic cavity mesenchyme adjacent to the heart, and allantoic mesenchyme, with transient detection in the ventral optic vesicle and surrounding frontonasal region (Fig. 4E–G). By E10.5, RALDH2 localization was observed in several trunk tissues, including somites, coelomic cavity mesenchyme, pericardium, cloaca, midgut, and mesonephric ducts (Fig. 4H). At E10.5 there was no longer an RALDH2 signal in the optic vesicles or frontonasal region (Fig. 4H).

Localization of ALDH1 and RALDH2 Proteins in Embryonic Neural Tissues

Immunohistochemical analyses of sections indicated that ALDH1 protein was clearly present in the midbrain localized to the ventral mesencephalon by E9.0 (data not shown) and continued to be localized there at E10.5, whereas RALDH2 protein was absent from this location (Fig. 5A,B). Neither ALDH1 nor RALDH2 were expressed in the forebrain, hindbrain, or dorsal midbrain through stage E10.5 (Fig. 4). Sections also demonstrated an abundance of ALDH1 protein in the dorsal retina at E11.5 (Fig. 5C) and in the lens plus the dorsal retina at E14.5 (Fig. 5D), but RALDH2 was not detected in either of these tissues (Fig. 5E, data not shown).ALDH1 protein was also localized in the medial aspect of the otic vesicles by E11.5, whereas RALDH2 was not detected in this tissue (Fig. 6A, B).

Although RALDH2 protein was not detected in cranial neural tissue following its disappearance from the optic vesicles at E9.5, nor in the neural tube up to E10.5, it was detected in trunk neural tissue later in development, particularly the spinal cord meninges and the motor neurons innervating the forelimbs at E12.5 (Fig. 5G). RALDH2 expression within the spinal cord motor neurons was limited to only the brachial and lumbar regions adjacent to the developing forelimbs and hindlimbs, respectively (data not shown). ALDH1 protein was not detected in the spinal cord (Fig. 5F).

ALDH1 and RALDH2 Protein Localization in Embryonic Thymus, Adrenal, and Metanephros

ALDH1, but not RALDH2, was detected in the thymic primordia at E9.5 (Fig. 4C) and was more abundant in this tissue at E10.5 (Fig. 4D). Serial sections provided evidence of ALDH1 protein localization bilaterally in the developing thymic primordia (third branchial pouches) at E11.5, with no detection of RALDH2 (Fig. 6A, B). Also, ALDH1 protein was localized to the adrenal blastemas at E11.5, the stage when this organ first develops, with RALDH2 once again being absent (Fig. 6C, D).

As described above, both ALDH1 and RALDH2 were found in the mesonephros by E10.5, suggesting a role in early genitourinary tract development. Examination of the metanephros (embryonic kidney) at E14.5 demonstrated RALDH2 protein in the developing renal cortex, with ALDH1 being nearly undetectable (Fig. 6E,F).

DISCUSSION

Regulated production of the ligand RA is undoubtedly a key event controlling the function of retinoid receptors during vertebrate development. Differences in the rate of RA synthesis from one embryonic tissue to another is likely to contribute to the establishment of RA gradients that affect the overall control of development by retinoids. Although it is clear that some embryonic tissues contain more RA than others, it is less clear how these differences are generated. Here we have explored the role of the aldehyde dehydrogenase gene family in embryonic RA synthesis. We found that mouse *Aldh1* and *Raldh2* both stimulate both stimulate RA synthesis when expressed in *Xenopus* embryos, with *Raldh2* being more efficient than *Aldh1*. The distinct localization patterns of ALDH1 and RALDH2 proteins in mouse embryos and adult tissues suggest that these two RA synthetic enzymes initially establish differential cranial and trunk RA synthesis and function in distinct retinoid signaling events during development.

Aldh1 and *Raldh2* Both Function in Embryonic RA Synthesis

We tested whether mouse *Aldh1* and *Raldh2*, as well as human *ALDH3*, could stimulate RA synthesis in vivo by ectopic expression in *Xenopus* embryos. Using the RA bioassay described here, endogenous RA is undetectable in *Xenopus* embryos from fertilization through blastula stage 8, thus providing a large time window during which embryos can be injected with ALDH mRNAs and examined for effects on RA synthesis with essentially no background detection. This assay further relies on the fact that *Xenopus* eggs and embryos contain large amounts of the vitamin A substrate retinal, the immediate precursor of RA

[Plack and Kon, 1961; Creech Kraft *et al.*, 1994; Blumberg *et al.*, 1996]. Based on our findings, we conclude that expression of either mouse *Aldh1* or *Raldh2* stimulates RA synthesis in this in vivo assay, but that expression of human *ALDH3* does not lead to this result. These observations demonstrate that the enzymes encoded by *Aldh1* and *Raldh2* can catalyze RA synthesis in *Xenopus* embryos using endogenous concentrations of substrate, coenzyme, and any other factors which may effect enzyme activity.

Our assays suggest that RALDH2 is more active than ALDH1 as a RA synthetic enzyme in *Xenopus* embryos. In vitro enzyme assays indicate that rat RALDH2 has a catalytic efficiency approximately 15-fold higher than that of rat ALDH1 [Wang *et al.*, 1996; Penzes *et al.*, 1997]. Thus, both physiological and biochemical analyses of ALDH1 and RALDH2 provide evidence that these enzymes indeed function as RA synthetic enzymes, with RALDH2 having higher activity. The lack of RA synthetic activity by human ALDH3 matches previous findings indicating that this enzyme lacks in vitro activity for oxidation of retinal to RA [Yoshida *et al.*, 1992]. This demonstrates that not all ALDHs are designed to function as RA synthetic enzymes.

Localization of ALDH1 and RALDH2 Proteins Relative to RA Distribution in Early Embryos

A posterior preference for initial RA synthesis during mouse embryogenesis has previously been reported [Hogan *et al.*, 1992], and may function to differentially regulate Hox genes, which help establish the anteroposterior axis during early development [Lufkin, 1996; Shimeld, 1996]. Previous studies on mouse embryos have shown that RA is initially present only in the trunk at E7.5–E8.0; from E8.5–E10.5 there is still a high level of RA in the trunk, but RA is now detectable in the optic vesicles and surrounding frontonasal region [Rossant *et al.*, 1991; Ang *et al.*, 1996a]. Here we found that the more active RA synthetic enzyme RALDH2 is initially localized in the trunk at E7.5–E8.0 and continues to be highly localized in trunk tissues from E8.5–E10.5 except transiently in the ventral optic vesicle and surrounding frontonasal region from E8.5–E9.5. Previous studies have shown that *Raldh2* mRNA is also localized primarily in trunk tissues from E7.5–E10.5, with transient expression in the optic vesicle region [Niederreither *et al.*, 1997]. In contrast, the less active RA synthetic enzyme ALDH1 is initially localized in cranial tissue (ventral mesencephalon) at E9.0 and then in several cranial tissues from E9.5–E10.5, including ventral mesencephalon, dorsal retina, otic vesicles, and thymic primordia. We previously reported that mouse *Aldh1* mRNA is detectable from E7.5 onwards [Ang and Duester, 1997], but since our present results show that ALDH1 protein is not detectable until E9.0, this enzyme is unlikely to contribute to RA synthesis until E9.0.

The expression patterns of the ALDH genes examined here are consistent with RALDH2 functioning to produce the RA seen in the trunk and frontonasal region, and with both RALDH2 and ALDH1 contributing to the RA observed in the optic vesicles. Indeed, *Raldh2* −/− knockout mouse embryos at late E8.5 (equivalent to our E9.0) exhibit a total elimination of RA detection in the trunk and frontonasal region, but some RA is still detectable in the optic vesicles [Niederreither *et al.*, 1999], perhaps produced by ALDH1. In addition, we suggest that ALDH1 may produce RA in several other cranial derivatives where this enzyme is detected, but where RA has not previously been detected, as discussed below.

Initial Localization of ALDH1 Is Primarily in Embryonic Cranial Derivatives

Previous studies indicated that embryonic dorsal retina (from E9.5 onwards) is a major site of protein accumulation for ALDH1 (formerly called AHD2) during mouse embryogenesis [McCaffery *et al.*, 1991]. There is convincing evidence that ALDH1 contributes to RA synthesis during retina development and in the adult retina [McCaffery *et al.*, 1991, 1993, 1999]. In those studies, the antibody used to localize ALDH1 was prepared against a phenobarbitol-inducible rat cytosolic ALDH [Lindahl and Evces, 1984] which is now known to be distinct from ALDH1, sharing 90% identity [Hsu *et al.*, 1999]. Evidently there was some cross-reaction of this rat ALDH-PB antibody with mouse ALDH1 in the dorsal retina, which contains particularly high levels of this enzyme, but other sites of ALDH1 localization may have been missed with this antibody. The mouse ALDH1 antibody we have described here does indeed detect ALDH1 in the dorsal retina as well as the lens, but our studies indicate that ALDH1 protein is also localized in several other cranial structures outside the eye not previously reported. Also, the mouse ALDH1 antibody described here does not cross-react with mouse ALDH-PB as described above.

We found localization of ALDH1 in the ventral aspect of the mesencephalic flexure from E9.0–E10.5. This finding suggests that the ventral mesencephalon may be a site of RA synthesis during brain development. However, analyses of transgenic RA reporter mouse embryos have not identified RA detection in the ventral mesencephalon [Rossant *et al.*, 1991; Balkan *et al.*, 1992]. If RA is indeed present in this tissue it may exist at a level too low to detect in these reporter mice or the reporter gene may be nonresponsive in the mesencephalon. Coinciding with the initial localization ofALDH1 at E9.0 in the ventral mesencephalon, this tissue initiates midbrain dopaminergic neuron development [Perrone-Capano and di Porzio, 1996], suggesting that ALDH1 may be functioning in this event. RA as well as retinoid receptors have been proposed to participate in dopamine signaling during late embryonic development and adulthood [Krezel *et al.*, 1998; Valdenaire *et al.*, 1998], and ALDH1 protein has been localized in axons and terminals of the mesostriatal and mesolimbic dopamine systems during late embryonic development and adulthood [McCaffery and Dräger, 1994a]. Interestingly, we previously reported that mouse *Adh4* (encoding a retinol dehydrogenase able to convert retinol to retinal) is also expressed in the ventral aspect of the mesencephalic flexure from E9.0–E10.5 [Haselbeck and Duester, 1998b]. This suggests that ADH4 and ALDH1 may cooperate to locally convert retinol to RA in the ventral mesencephalon at the time when midbrain dopaminergic neurons first develop, but as of yet a direct demonstration of the presence of RA in this location is lacking.

The otic vesicles were found to contain ALDH1 protein at stages E10.5–E11.5. Expression was observed to be asymmetric, with immunodetection occurring only along the medial portion of the vesicle. *Adh4* expression is also observed along the medial portions of the otic vesicles [Haselbeck and Duester, 1998b]. Since a role for RA in the development of the inner ear has been proposed [Corey and Breakefield, 1994], it is reasonable to propose that ADH4 and ALDH1 may function to locally convert retinol to RA for otic vesicle differentiation. However, transgenic RA reporter mouse embryos do not have RA detection

in the otic vesicles [Rossant *et al.*, 1991; Balkan *et al.*, 1992], suggesting that the RA level may be low or that the reporter gene is nonresponsive in this tissue.

The thymic primordia develop as two symmetrical epithelial elongations in the ventral portion of the third brachial pouch, which then migrate caudally and fuse to form the thymus gland [Smith, 1965; Cordier and Haumont, 1980]. Our detection of ALDH1 protein, but not RALDH2 protein, in the thymic primordia from E9.5–E11.5 suggests that ALDH1 may catalyze local RA synthesis needed for thymus development. As the developing thymic primordia lie close to the boundary with trunk tissues that clearly contain RA, as observed in transgenic RA reporter mouse embryos, it is unclear if the thymic primordia themselves have detectable RA in such reporter embryos [Rossant *et al.*, 1991; Balkan *et al.*, 1992]. However, analyses of RAR null mutant mice have demonstrated the existence of persistent cervical thymus and other thymic abnormalities, indicating that retinoid signaling is required for proper development and caudal migration of the thymus [Mendelsohn *et al.*, 1994].

Initial Localization of RALDH2 Is Primarily in Embryonic Trunk Tissues

Our immunohistochemical observations of RALDH2 protein localization in mouse embryos from E7.5–E10.5 correlate well for the most part with the results found previously for *Raldh2* mRNA expression by in situ hybridization [Hiederreither *et al.*, 1997]. From E7.5–E10.5, RALDH2 protein is quite widespread in many trunk tissues, such as paraxial mesoderm, somites, pericardium, coelomic cavity mesenchyme, and mesonephros and is absent from cranial tissues with the exception of transient expression in the ventral optic vesicles and frontonasal region. Examination of *Raldh2* −/− mice has demonstrated that RALDH2 is in fact necessary for development of many trunk structures, including somites, heart, limb buds, and mesonephric ducts, but cranial defects are also seen, including truncation of the frontonasal region and reduction in size of the otic vesicles [Niederreither *et al.*, 1999]. RALDH2 protein localization in the frontonasal region from E8.5–E9.5 could account for its involvement in RA production for frontonasal development. *Raldh2* mRNA was reported in the lateral regions of the otic vesicles at E10.5 [Niederreither *et al.*, 1997], which could account for local RA synthesis, but we observed no RALDH2 protein localization in the otic vesicles by this stage.

A further indication that RALDH2 functions to produce RA for trunk tissues comes from studies on the spinal cord where RALDH2, but not ALDH1, is localized. At E12.5 we found that RALDH2 protein localizes in the motor neurons which innervate the forelimbs and hindlimbs, but not in other regions of the spinal cord. Previous studies have shown that *Raldh2* mRNA is highly localized by E12.5 of mouse development in a subpopulation of motor neurons innervating the limbs [Zhao *et al.*, 1996], a pattern which is consistent with higher levels of RA in the brachial and lumbar regions of the embryonic spinal cord [McCaffery and Dràger, 1994b], and higher levels of retinoid-X receptor signaling in these regions [Solomin *et al.*, 1998]. Furthermore, ectopic expression of *Raldh2* in the chick spinal cord affects the specification of motor neuron subtype identity [Sockanathan and Jessell, 1998]. Together, these facts provide a compelling case for *Raldh2* functioning to provide RA for a unique retinoid signaling event which specifies the identity of spinal cord motor neurons innervating the limbs.

Localization of ALDH1 and RALDH2 in Embryonic Mesonephros, Metanephros, and Adrenal

The high level of RALDH2 protein initially observed in the somites is much reduced by E10.5, but at this stage there is a high level of RALDH2 protein localized along the entire anteroposterior axis of the coelomic cavity and mesonephric ducts. *Adh1*, encoding a retinol dehydrogenase, is also expressed along the entire anteroposterior axis of the mesonephric ducts at both the mRNA level [Ang *et al.*, 1996a] and the protein level (R.J. Haselbeck and G. Duester, unpublished data), thus potentially setting up a metabolic pathway to convert retinol to RA in the mesonephros. Interestingly, ALDH1 protein is also present in the mesonephric ducts at E10.5, but it is unevenly distributed along the anteroposterior axis such that localization is highest just posterior to the forelimb and hindlimb buds. This mesonephric pattern of ALDH1 localization could help boost the level of RA diffusing into the posterior regions of developing limb buds; however it is clearly not sufficient to support limb development, as *Raldh2* knockout mice do not form limb buds [Niederreither *et al.*, 1999].

It is likely that mesonephric localization of both ALDH1 and RALDH2 also provides the RA known to be needed for correct development of mesonephric derivatives of the genitourinary tract, which are sensitive to vitamin A deficiency or RAR null mutations [Wilson *et al.*, 1953; Mendelsohn *et al.*, 1994]. *Raldh2* −/− mice lack mesonephric ducts, indicating that RA is needed for their initial development [Niederreither *et al.*, 1999]. Examination of RAR null mutations have shown that the metanephros also does not develop properly when retinoid signaling is disrupted [Mendelsohn *et al.*, 1994]. At E14.5, our studies indicate that RALDH2, but not ALDH1, is abundant in the metanephros. We have previously shown that *Adh1* is expressed in the metanephros at E16.5 [Ang *et al.*, 1996b], thus suggesting that ADH1 and RALDH2 may cooperate to convert retinol to RA in this organ.

Previous immunohistochemical studies have shown that ADH1 and ADH4 (both retinol dehydrogenases) localize to the adrenal blastema is it first develops [Haselbeck and Duester, 1998a], and that RA is detectable in isolated adrenal glands at stage E16.5 [Haselbeck *et al.*, 1997]. Here we demonstrate that ALDH1 protein also localizes to the adrenal blastema, thus providing evidence that enzymes needed for both steps of the metabolic pathway converting retinol to RA exist in the adrenal gland.

Unique Functions for *Aldh1* and *Raldh2* in Retinoid Signaling

The bewildering array of retinoid signaling events which have been described over the years require intricately regulated pathways to supply RA at just the right time, place, and amount. The different efficiencies with which *Aldh1* and *Raldh2* are able to stimulate RA synthesis in *Xenopus* embryos, as well as their unique expression patterns in mouse embryos, suggests that these genes control distinct retinoid signaling pathways. Whereas *Raldh2* undoubtedly functions as the predominant regulator of RA synthesis in midgestation mouse embryos, our findings suggest that *Aldh1* is also likely to regulate embryonic RA synthesis in several discrete locations requiring low levels of RA. Also, the widespread occurrence of ALDH1,

but not RALDH2, proteins in adult mouse organs argues that *Aldh1* may function as the predominant regulator of RA synthesis in the adult organism.

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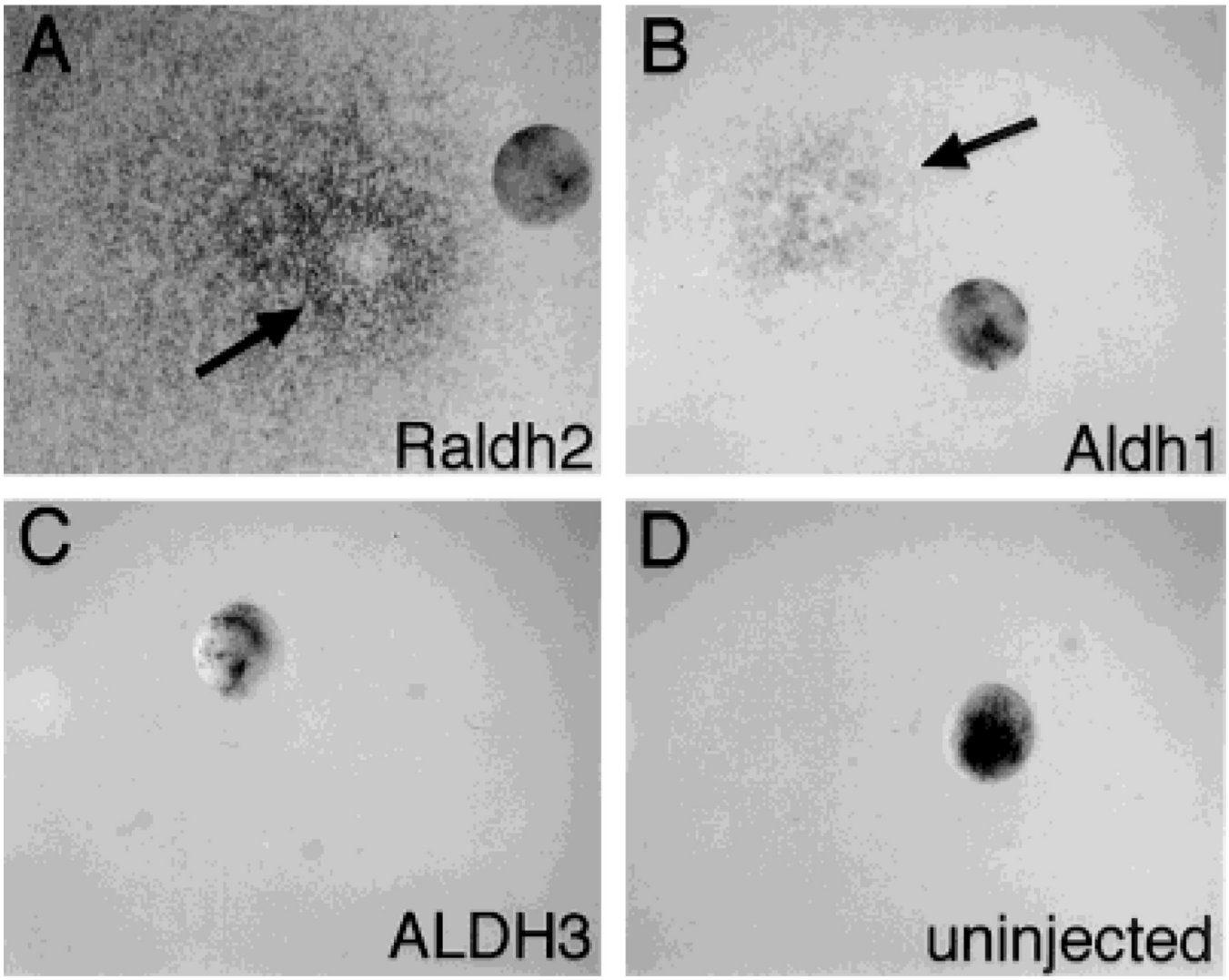

Fig. 1.

Aldh1 and *Raldh2* mRNA transcripts stimulate RA synthesis in *Xenopus* embryos. *Xenopus* embryos at the 2–4 cell stages were injected with 23 nl of mRNA(0.2 μg/μl) transcribed from either mouse *Raldh2* (**A**), mouse *Aldh1* (**B**), or human *ALDH3* (**C**), or were uninjected as a control (**D**). Following growth to blastula stage 8, embryos were subjected to an RA bioassay which involves incubating the embryo on a lawn of RA-reporter cells followed by assay for *lacZ* expression. In each photograph the embryo has been moved aside from its site of incubation, and arrows point to sites of *lacZ* expression.

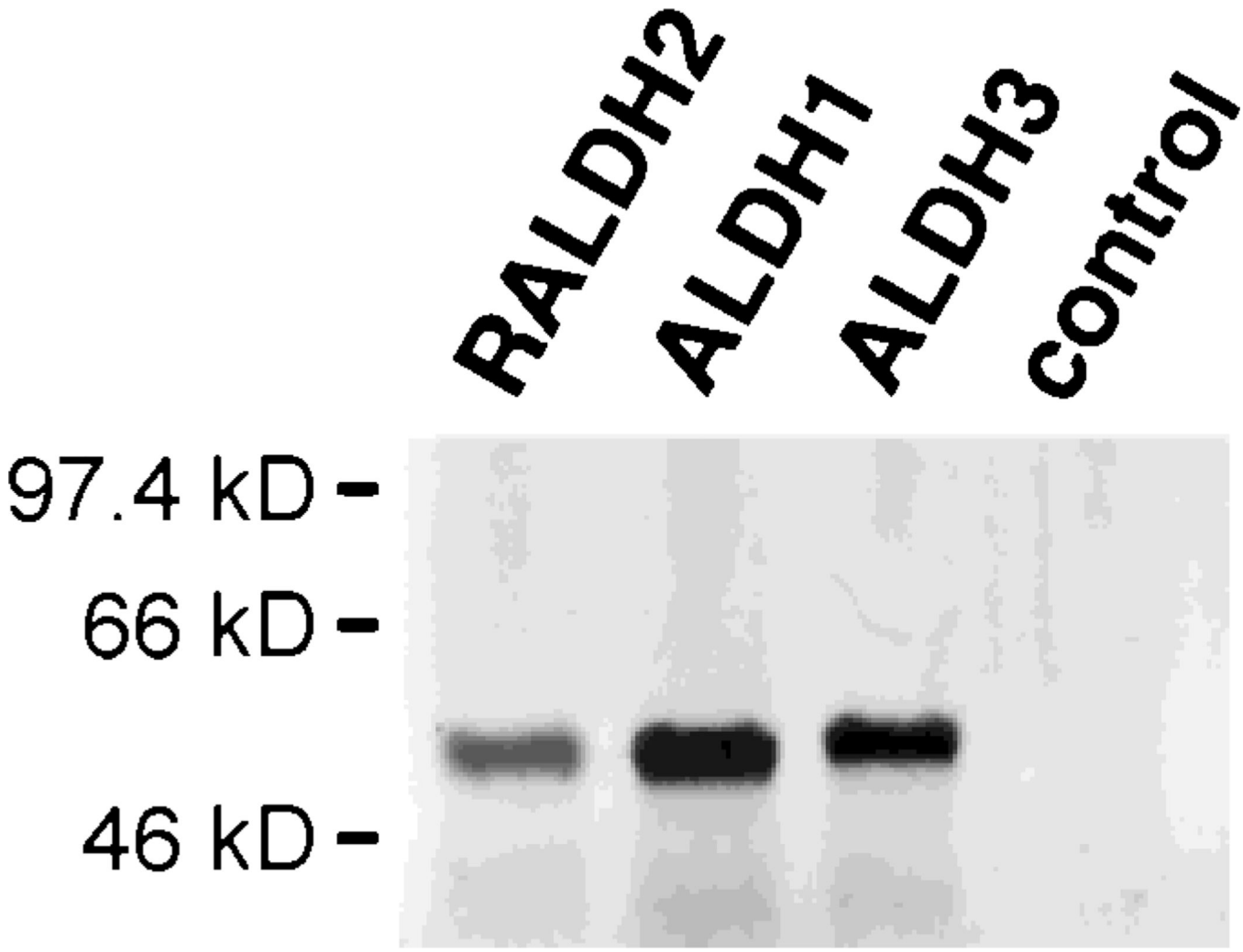

Fig. 2.

Relative efficiency of protein production from ALDH mRNAs. Mouse *Raldh2*, mouse *Aldh1*, and human *ALDH3* mRNA samples were examined for the ability to produce protein by translation of 0.4 μg of each sample in the presence of [35S]methionine followed by SDS-polyacrylamide gel electrophoresis. Each mRNA produced a protein of approximately 55 kDa, the expected size of an ALDH subunit, and a control sample containing no mRNA produced no protein.

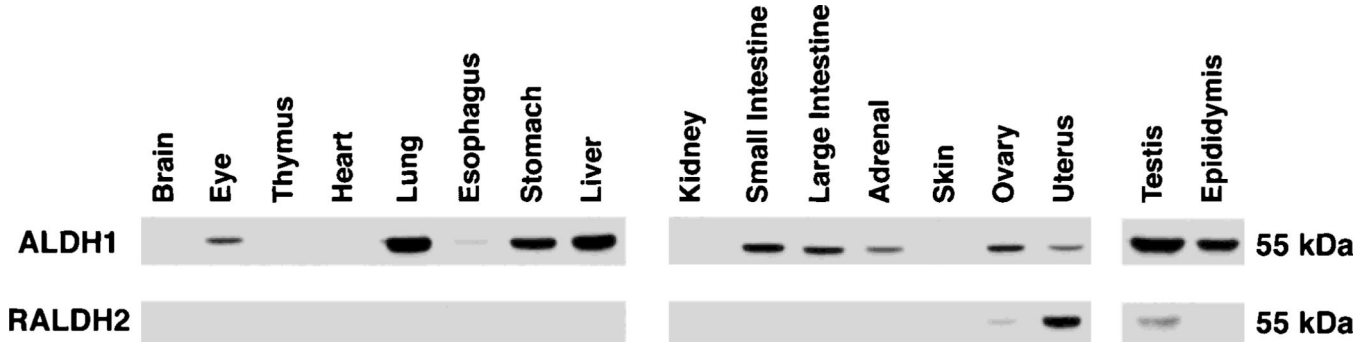

Fig. 3.

Western blot of adult mouse tissues showing specificity of antibodies for ALDH1 and RALDH2 proteins. Tissue extracts were loaded at 20 μg of total protein per lane. Blots were sequentially probed with each antibody, and proteins detected were 55 kDa in each case.

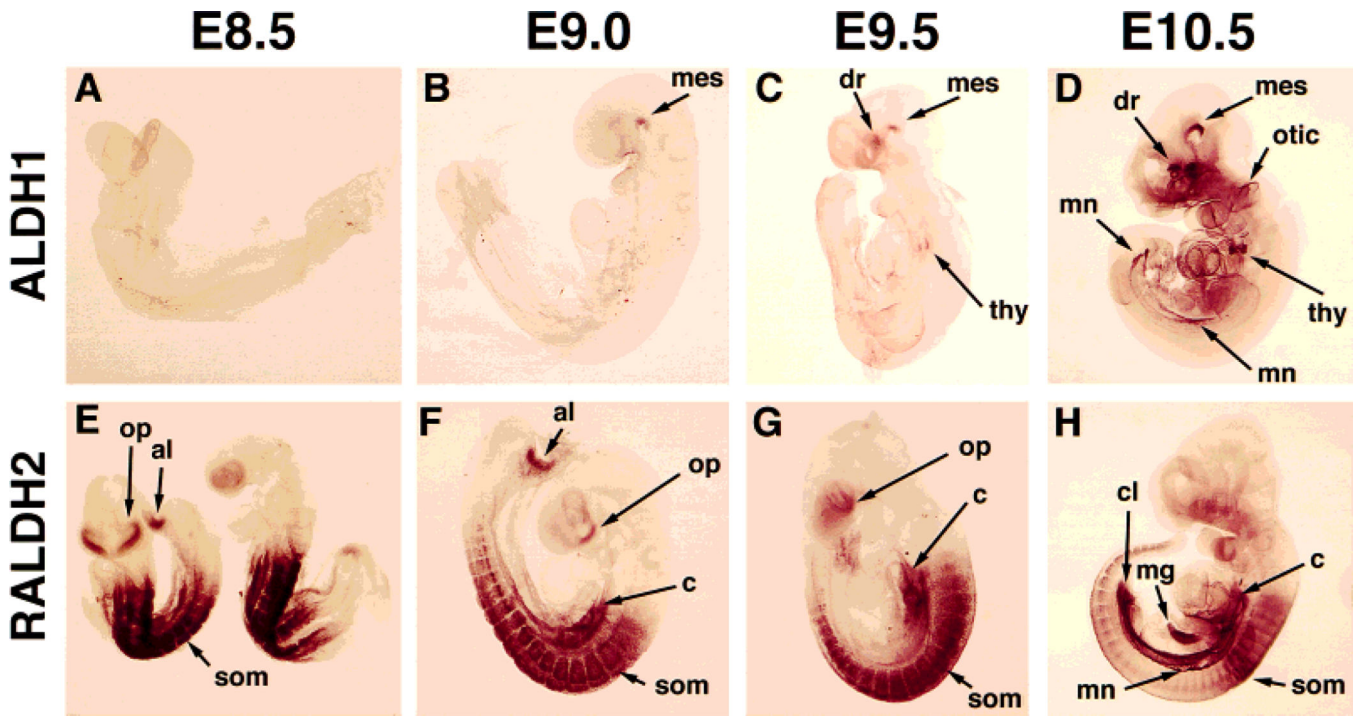

Fig. 4.

Localization of ALDH1 and RALDH2 proteins during stages E8.5–E10.5 of mouse embryogenesis. Embryos were subjected to whole-mount immunohistochemistry and prior to photography were cleared in benzyl alcohol:benzyl benzoate (1:2). Due to the transparent nature of the cleared embryos, both portions of bilateral structures are observed simultaneously. al, allantoic mesenchyme; c, coelomic cavity mesenchyme; cl, cloaca; dr, dorsal retina; mes, ventral flexure of mesencephalon; mg, midgut; mn, mesonephric ducts; op, optic vesicle/frontonasal mass; otic, otic vesicle; som, somite; thy, thymic primordia.

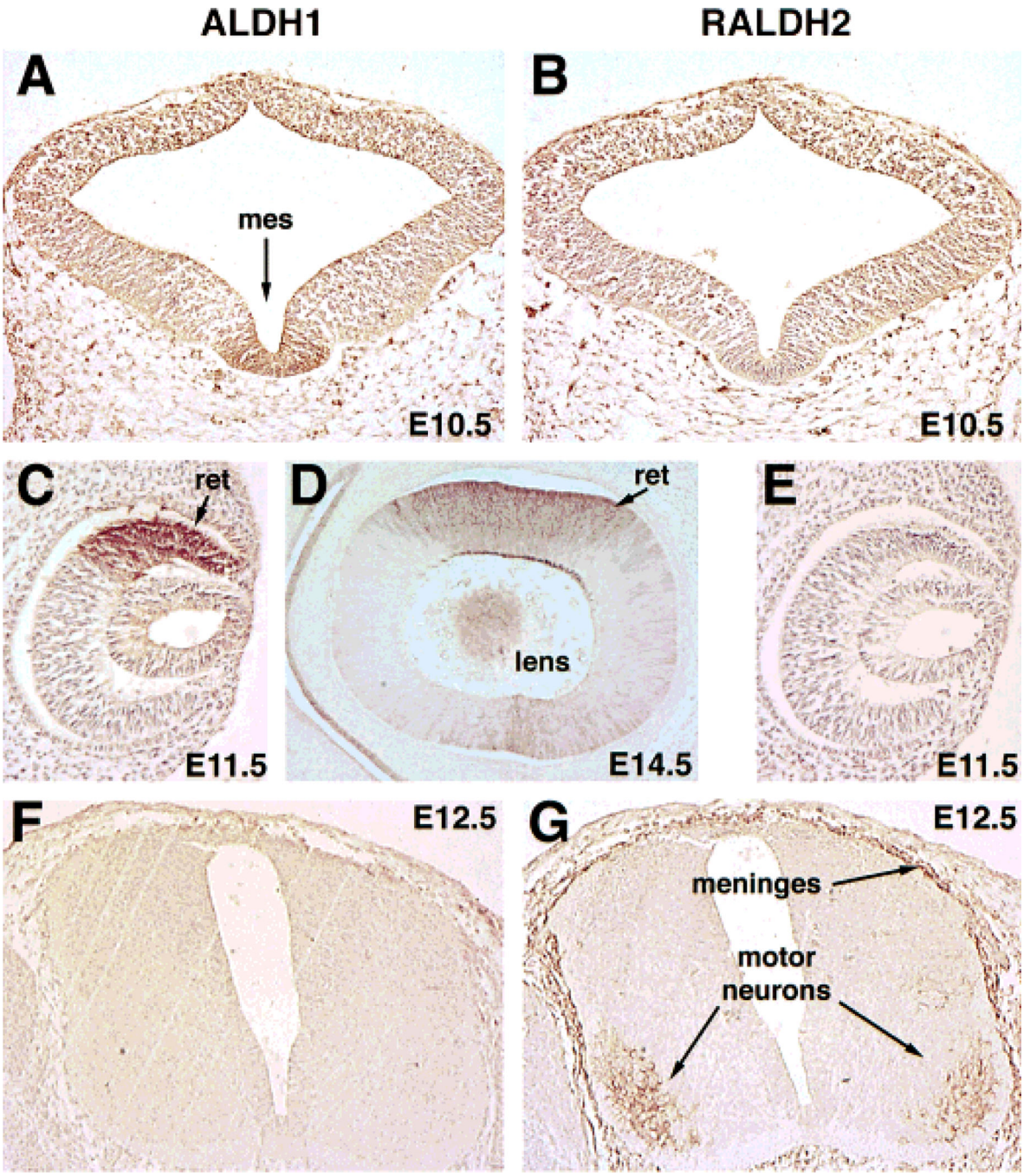

Fig. 5.

Immunohistochemical localization of ALDH1 and RALDH2 proteins in neural tissues. In the mesencephalic ventral flexure (mes) immunohistochemical analyses of E10.5 embryos sectioned coronally shows the presence of ALDH1 (**A**) but not RALDH2 (**B**). In coronal sections through the eye, ALDH1 immunodetection occurs in the dorsal retina (ret) at E11.5 (**C**) and in both dorsal retina and the lens at E14.5 (**D**), with no detection of RALDH2 (**E**). In transverse sections through the brachial spinal cord at E12.5, ALDH1 protein is absent (**F**), but RALDH2 protein is detected in the motor neurons and meninges (**G**).

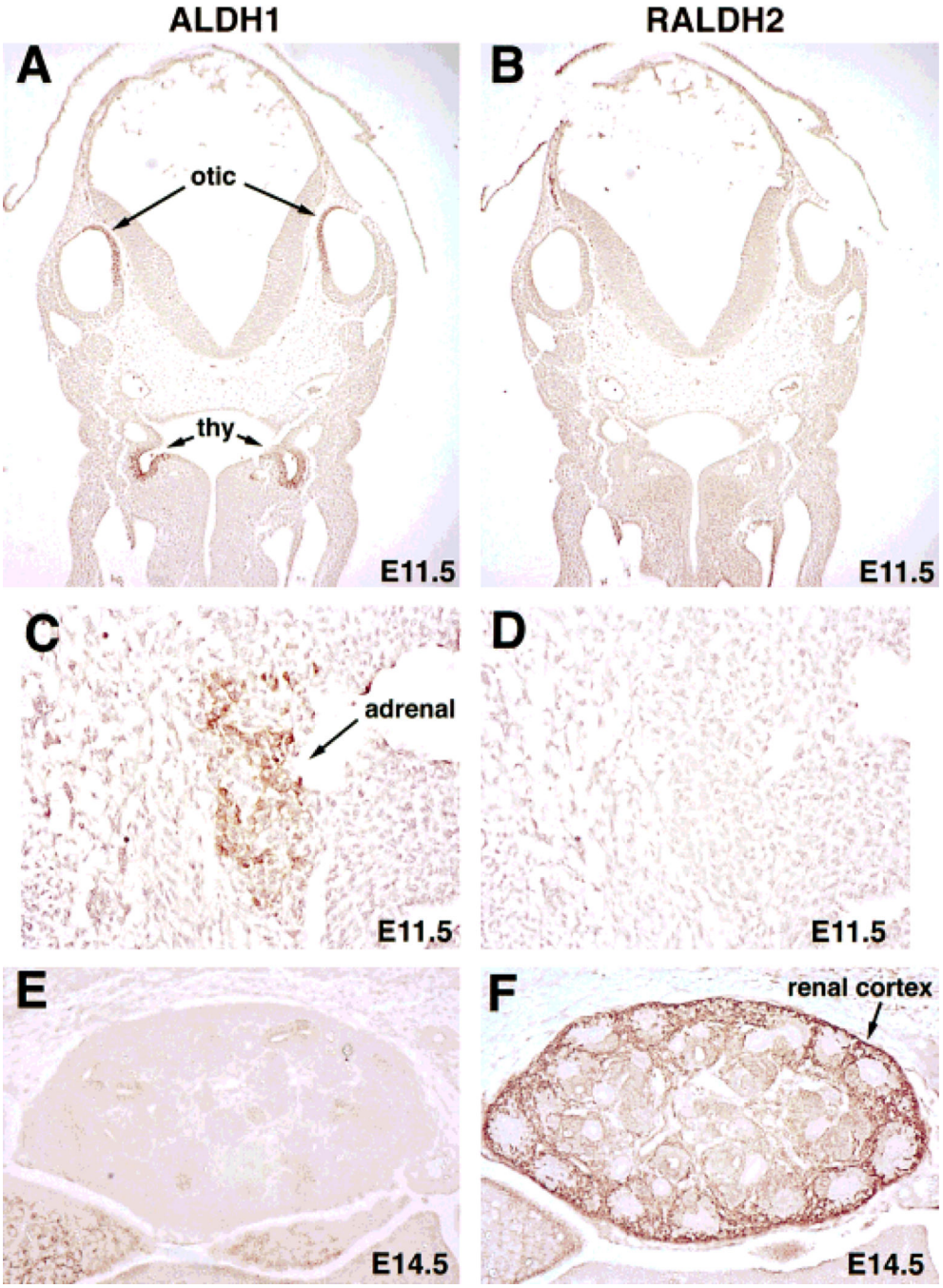

Fig. 6.

Organ-specific localization of ALDH1 and RALDH2 proteins revealed by immunohistochemistry. Analyses of E11.5 embryos sectioned coronally demonstrates ALDH1 protein in the medial portions of the otic vesicles and bilaterally in the thymic primordia (thy) (**A**), whereas RALDH2 is absent in these tissues (**B**). In sagittal sections of the E11.5 adrenal blastema, ALDH1 protein is detected (**C**), but not RALDH2 protein (**D**). At E14.5, sagittal sections through the developing kidney shows that ALDH1 protein is

absent (**E**), but that RALDH2 protein is abundant and localized primarily in the renal cortex (**F**).

TABLE 1

ALDH1 and RALDH2 Immunohistochemical Localization During Mouse Embryogenesis

Stage/Tissue	ALDH1	RALDH2
E7.5–E8.0		
Anterior (headfolds)	−	−
Posterior (paraxial mesoderm)	−	+
E8.5		
Neural folds/tube	−	−
Optic vesicle	−	+
Otic vesicle	−	−
Somites	−	+
Paraxial mesoderm	−	+
Coelomic cavity adjacent to heart	−	+
Allantoic mesenchyme	−	+
E9.0–E10.5		
Cranial		
Forebrain	−	−
Midbrain (mesencephalon)	+	−
Hindbrain	−	−
Optic vesicle (dorsal retina)	+	−
Optic vesicle (ventral/frontonasal)	−	+
Otic vesicle	+	−
Thymic primordia	+	−
Trunk		
Neural tube	−	−
Somites	−	+
Limb buds	−	+
Coelomic cavity mesenchyme	−	+
Pericardium	−	+
Cloaca	−	+
Midgut	−	+
Mesonephros	+	+