Retinoic Acid Synthesis in Mouse Embryos during Gastrulation and Craniofacial Development Linked to Class IV Alcohol Dehydrogenase Gene Expression*

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Endogenous retinoic acid (RA) has been observed in vertebrate embryos as early as gastrulation, but the mechanism controlling spatiotemporal synthesis of this important regulatory molecule remains unknown. Some members of the alcohol dehydrogenase (ADH) family catalyze retinol oxidation, the rate-limiting step in RA synthesis. Here we have examined mouse embryos for the presence of endogenous RA and expression of ADH genes. RA was not detected in egg cylinder stage embryos but was detected in late primitive streak stage embryos. Detection of class IV ADH mRNA, but not class I or class II, coincided with the onset of RA synthesis, being absent in egg cylinder embryos but present in the posterior mesoderm of late primitive streak embryos. During neurulation, RA and class IV ADH mRNA were colocalized in the craniofacial region, trunk, and forelimb bud. Class IV ADH mRNA was detected in cranial neural crest cells and craniofacial mesenchyme as well as trunk and forelimb bud mesenchyme. The spatiotemporal expression pattern and enzymatic properties of class IV ADH are thus consistent with a crucial function in RA synthesis during embryogenesis. In addition, the finding of endogenous RA and class IV ADH mRNA in the craniofacial region has implications for the mechanism of fetal alcohol syndrome.

Retinoic acid (RA), a metabolite of vitamin A (retinol), is known to regulate differentiation during vertebrate embryogenesis (Maden, 1994; Hofmann and Eichele, 1994) by acting as a ligand controlling the activity of the retinoic acid receptor (RAR) family of transcriptional regulators (Kastner et al., 1994; Mangelsdorf et al., 1994). Studies on mice carrying RAR null mutations have shown that RA plays a crucial role in the development of the craniofacial region (cranial neural crest, skeleton, and numerous organs (Lohnes et al., 1994; Mendelsohn et al., 1994). Many of the defects noticed in RAR mutants are also present in vitamin A-deficient vertebrate embryos (Wilson et al., 1953; Dersch and Zile, 1993). Information on the presence of endogenous RA during development is available from a variety of vertebrate embryos. In avian and amphibian embryos RA has been detected during gastrulation in Hensen’s node and Spemann’s organizer, respectively, (Chen et al., 1992, 1994; Twal et al., 1995) as well as in the forelimb bud (Thaller and Eichele, 1987; Scadding and Maden, 1994) and floor plate of the neural tube (Wagner et al., 1990). In mammalian embryos RA has been detected in the retina (McCaffery et al., 1993), spinal cord (Wagner et al., 1992; McCaffery and Dräger, 1994; Horton and Maden, 1995), and forelimb bud (Scott et al., 1994; Horton and Maden, 1995). Gastrulation stage mouse embryo tissues are capable of RA synthesis, occurring preferentially in the node (Hensen’s node equivalent) and primitive streak (Hogan et al., 1992). The involvement of RA in mouse cranial neural crest development has been inferred from studies on the teratogenic effects of RA excess (Morriss-Kay, 1993) as well as RAR mutations (Lohnes et al., 1994). These findings suggest that synthesis of RA from retinol may be regulated spatially and temporally in the developing embryo, thus providing a mechanism to differentially activate the RA receptors.

Retinol, like other alcohol compounds, is converted by a two-step oxidative process to an aldehyde, retinal, and then to a carboxylic acid, RA, with the first reaction representing the rate-limiting step (Kim et al., 1992; Blaner and Olson, 1994). While members of the alcohol dehydrogenase enzyme family have been implicated as catalyzing the second step of RA synthesis in early mouse embryos (McCaffery et al., 1992, 1993; McCaffery and Dräger, 1994), the physiologically relevant catalyst for the first step, the oxidation of retinol, in early embryonic tissues remains obscure. The enzyme responsible for a retinol dehydrogenase activity purified from the adult mouse epidermis (Connor and Smit, 1987) was found to be identical to a cytosolic medium chain alcohol dehydrogenase (ADH) previously purified from the mouse stomach (Algar et al., 1983), now identified by our laboratory as class IV ADH (Zgombić-Knight et al., 1995). Evidence has also been presented that a microsomal retinol dehydrogenase exists and that the adult rat liver form is a member of the short chain dehydrogenase/reductase family (Chai et al., 1995). The ADH and short chain dehydrogenase/reductase enzyme families are related evolutionarily, sharing a similar coenzyme-binding domain, but differ in that ADH has a greater subunit molecular weight and is zinc-dependent, whereas the other family has a shorter subunit and no metal requirement (Persson et al., 1991). Whereas both the ADH and short-chain dehydrogenase/reductase families are now known to contain retinol dehydrogenases expressed in adult tissues, an embryonic retinol dehydrogenase associated with early RA synthesis has not previously been described.

The ADH family contains the classical liver ADH (now known as class I ADH) responsible for ethanol metabolism in vertebrates, as well as several other classes that preferentially oxidize alcohols other than ethanol (Danielsson et al., 1994). Five classes of enzymes have been identified in the human ADH gene family, but we have shown that the mouse ADH gene family contains only three of these classes, i.e. genes encoding ADH classes I, III, and IV (Zgombić-Knight et al., 1994).
1995). In particular, the conserved human and rodent class IV ADHs have been found to have a higher specificity for retinol than other members of the ADH family (Boleda et al., 1993; Yang et al., 1994). We have also demonstrated that mouse class IV ADH is expressed in a wide variety of epithelia, the main retinoid target tissue of adults, further suggesting that retinol oxidation may be a primary function of this enzyme (Zgombic-Knight et al., 1995). In this study we have compared the presence of endogenous RA in mouse embryos with the expression of all three classes of mouse ADH during early embryogenesis and have found that class IV ADH mRNA colocalizes with RA.

EXPERIMENTAL PROCEDURES

Staging of Embryos—Embryos (mouse strain FVB/N) were staged by vaginal plug appearance with noon on the day of plug detection being considered 0.5 days post-coitum (dpc). Further staging of mouse embryos was according to Kaufman (Kaufman, 1992).

Retinoic Acid Bioassay—The presence of RA was monitored by culturing whole mouse embryos or dissected embryonic tissues as explants on top of a monolayer of RA reporter cells. The RA reporter cell line F9-RARE-lacZ (Sil-15) was used as a bioassay to monitor the diffusion of RA from embryonic tissues as described previously (Wagner et al., 1992). This cell line was derived from stable transfection of mouse F9 teratocarcinoma cells with a transposon consisting of a promoter with a RA response element driving the expression of lacZ encoding β-galactosidase. The RA response element used in this reporter cell line is activated by RA but not by precursor retinoids such as retinol or retinal. As indicated previously, this assay is optimal for detection of all-trans-RA, but will also detect the 9-â and 13-â isomers with about a 100-fold lower efficiency (McCaffery and Dräger, 1994). Other biologically active retinoid carboxylates, such as dihydro-RA and 4-oxo-RA, have not been tested but may also stimulate transcription of the RARE-lacZ reporter gene used here. Thus, the results presented here reflect the combination of all biologically active forms of RA in the tissues examined.

Cell stocks were maintained at 37 °C and 5% CO₂ in 0.1% gelatin-coated plates in L15 CO₂ tissue culture medium with FVM and 1:1:2 supplements containing antibiotics (Specialty Media, Inc., Lavallette, NJ), 10% fetal calf serum, and 0.2 mg/ml G418. In our hands the F9-RARE-lacZ cells were found to be sensitive to subnanomolar concentrations of RA; β-galactosidase activity was detected in control F9-RARE-lacZ cells incubated with as little as 0.1 nM RA added to the above media, and there was virtually no background detection of β-galactosidase in cells incubated without added RA.

For tissue explant studies, F9-RARE-lacZ cells were grown in gelatin-coated 24-well plates in the above media without G418 until 80–90% confluent, at which time embryonic tissues were placed on top of the monolayer and incubated for 18 h. Whole embryos were dissected away from the decidua and extraembryonal cone in tissue culture medium prior to culturing as explants. Following incubation, the reporter cells were fixed in 1% glutaraldehyde, and β-galactosidase activity was visualized with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as described (Lim and Chae, 1989). This colorimetric assay enabled detection of RA that had been released from the tissue explant and diffused to the reporter cells. In many cases the embryonic tissue remained fixed to the reporter cells, allowing a determination of differential release of biologically active forms of RA from various portions of the embryo.

Whole-mount In Situ Hybridization—Mouse embryos from 6.5–9.5 dpc were fixed and subjected to whole-mount in situ hybridization using antisense riboprobes according to Wilkinson (1992). Antisense riboprobes for mouse class I ADH, class III ADH, and class IV ADH genes were prepared from full-length cDNAs; previous studies indicated a lack of cross-hybridization between these three mouse ADH genes, which share about 60% sequence identity evenly spread out over their entire coding regions (Zgombic-Knight et al., 1995). A sense riboprobe derived from a full-length class IV ADH DNA was used as a control to monitor nonspecific detection. All probes were hybridized under identical conditions. Under the conditions used here, nonspecific detection using the control sense riboprobe was not observed in 6.5–8.5 dpc embryos and was minimal in 9.5 dpc embryos. The lack of nonspecific detection was also confirmed using the antisense riboprobe for the class I ADH gene, which was not expressed from 6.5–8.5 dpc and thus gave results indistinguishable from the control sense riboprobe.

Embryo Sectioning—Following whole-mount in situ hybridization, some embryos were embedded in paraffin and sectioned at 14 μm using standard methodology (Kaufman, 1992). Sections of class I ADH-hybridized embryos were counterstained with neutral red, and sections of class IV ADH-hybridized embryos were not counterstained.

RESULTS

Identification of Endogenous Retinoic Acid in Mouse Embryos—We monitored the presence of RA in individual mouse embryos by culturing them as explants on top of a monolayer of RA reporter cells that employ a RA response element joined to lacZ as the reporter gene (Wagner et al., 1992). This served as a bioassay to detect the presence of RA released from the embryo as detected by β-galactosidase activity in the surrounding RA reporter cells. We observed no RA release from embryos at 6.5 dpc during the egg cylinder stage (0 out of 25 examined), thus indicating that RA does not exist or is too low to detect at this stage (Fig. 1A). In contrast, RA release was detected in 7.5 dpc embryos (63 out of 66 examined), suggesting that late primitive streak stage embryos undergoing gastrulation are competent to synthesize RA (Fig. 1, B and C). RA release occurred along the surface of the embryonic tissues but not along the extraembryonic tissues (Fig. 1, B and C).

When 8.5 dpc embryos undergoing neurulation were cultured on the RA reporter cells they underwent the characteristic axial rotation sequence (to attain the characteristic fetal position), and almost all embryos (24 out of 25 analyzed) were observed to release large amounts of RA from the trunk region as well as smaller amounts of RA from the ventral cephalic region (Fig. 1, D and E). The cephalic site of RA detection corresponds to the optic eminences, which at this stage contain the optic vesicles as well as surrounding periorbital mesenchyme derived from the cranial neural crest. Thus, in 8.5 dpc embryos a preference for RA release from posterior tissues derived from the primitive streak was observed, but RA was also detected in the cranial region.

Embryos at 9.5 dpc incubated on the RA reporter cells (12 out of 12 examined) also released RA in large amounts from the trunk and smaller amounts from the head. The maximum amount of RA release noted in the trunk was centered in the prospective forelimb bud region, with less RA release in the posterior trunk (Fig. 1F). In the head, maximum RA release was observed in the vicinity of the craniofacial region (optic eminences) with much less RA detected in the posterior hindbrain (Fig. 1F). We also examined RA release from 9.5 dpc head tissue dissected anterior to the first branchial arch and found that RA was localized to the craniofacial region but was absent from the anterior hindbrain (6 out of 6 examined) (Fig. 1, G and H). Upon further dissection we observed that RA was indeed released from cranial tissue including the optic eminences (3 out of 3 examined) (Fig. 1I), but not from tissue containing the posterior midbrain-anterior hindbrain (0 out of 3 examined) (Fig. 1J).

RA was also easily detected in dissected 9.5 dpc forelimb bud tissue incubated on the RA reporter cells (18 out of 18 examined); no anterior-posterior difference was noted, and the limb buds were not large enough to determine if a proximal-distal difference existed (Fig. 1K). Some forelimb buds from 10.5 dpc embryos were observed to release small amounts of RA (4 out of 12 examined) and were large enough to determine that RA was released from only from the proximal side near the attachment to the trunk, and equally along the anterior-posterior axis (Fig. 1L).

Class IV ADH Gene Expression in Mouse Embryos—To examine the potential role of class IV ADH in early embryonic RA synthesis, we used whole-mount in situ hybridization to test whether its gene was expressed in 6.5–9.5 dpc mouse embryos during the time our RA measurements suggested that RA synthesis commences. Class IV ADH mRNA was not detected
FIG. 1. Class IV ADH and Embryonic Retinoic Acid Synthesis
at 6.5 dpc during the egg cylinder stage (Fig. 2A). Class IV ADH mRNA was first detected during gastrulation at 7.0–7.5 dpc in the posterior region of the embryo along the primitive streak from its anterior end (node) to where it joins the allantois posteriorly (Fig. 2, B and C). No expression was detected in the extraembryonic tissues. Sections of whole-mount stained embryos indicated that mRNA was primarily localized to the primitive streak mesoderm (Fig. 3A).

During neurulation at 8.0 dpc, class IV ADH mRNA was observed anteriorly in the neural folds as well as posteriorly in the primitive streak (Fig. 2, D and E). Class IV ADH mRNA in the neural folds of 8.0 dpc embryos was localized to the dorsolateral region of the neuroepithelium corresponding to the site of cranial neural crest cell emigration; mRNA was also observed in the cranial mesenchyme, which consists primarily of neural crest cells that have migrated out of the neuroepithelium and undergone an epithelial-mesenchymal transition (Fig. 3B). At 8.5–9.0 dpc, class IV ADH mRNA was detected in the dorsolateral regions of the neural folds and craniofacial region, as well as in the trunk and caudal neuropore (Fig. 2, F and G). Sections at this stage indicated that class IV ADH mRNA was localized primarily to the periorbit neural crest mesenchyme as well as the first and second branchial arches derived from hindbrain neural crest emigration (Fig. 3C).

At 9.5 dpc class IV ADH mRNA in the head was limited to the ventral craniofacial structures (frontonasal mass, periorbit region, mandibular and maxillary components of the first branchial arch, and the second branchial arch) but was absent or low in all regions of the brain (Fig. 2H). Sections of stained embryos indicated that the class IV ADH mRNA signal was greater in the mesenchyme of the frontonasal, periorbit, and branchial arch regions and close to background in the neuroepithelium of all regions of the brain as well as the optic vesicle (Fig. 3D). At 9.5 dpc class IV ADH mRNA was also detected in the posterior trunk and forelimb bud (Fig. 2H). mRNA in the trunk was primarily localized to paraxial mesoderm, particularly in the most posterior portion of the trunk where somites had not yet formed, as well as in the somites posterior to the forelimb bud (Fig. 3E). Class IV ADH mRNA in the forelimb bud was localized primarily to proximal rather than distal mesenchyme, and no anterior-posterior difference was noted (Fig. 3F).

Expression of Class I and Class III ADH Genes in Mouse Embryos—The above findings indicate that class IV ADH gene expression corresponds both temporally and spatially with the presence of RA during early embryogenesis. In contrast, expression of the two other known mouse ADH genes (encoding the class I and class III forms) detected by whole-mount in situ hybridization was quite different than that observed for the class IV ADH gene. Class I ADH mRNA could not be detected in embryos from 6.5 to 8.5 dpc (Fig. 4, A–C). However, mRNA was detected at 9.5 dpc within the trunk region (Fig. 4D), and this was localized to the mesonephros in sections of stained embryos (Fig. 3, G and H). Class III ADH mRNA was observed nearly ubiquitously from 6.5 to 9.5 dpc (Fig. 4, E–H); this was confirmed by analysis of sections (data not shown). Thus, class I and class III ADH gene expression did not correlate with the spatiotemporal appearance of RA as detected with our bioassay.

DISCUSSION

The present results show that embryonic RA synthesis is not yet occurring at the egg cylinder stage (6.5 dpc) but that it commences during the primitive streak stage (i.e. at least by 7.5 dpc). Our findings thus suggest that RA first plays a role in embryogenesis during gastrulation, consistent with two earlier studies addressing the spatial and temporal initiation of RA synthesis during gastrulation. First, expression of a lacZ transgene linked to an RA response element is initially detected at 7.5 dpc limited to the primitive streak of mouse embryos (Rossant et al., 1991). Second, it has been demonstrated that cultured posterior tissues (primitive streak and node) of 7.75 dpc mouse embryos are more competent than anterior tissues (headfold) to convert labeled retinol to RA (Hogan et al., 1992). Thus, our bioassay supports the previously held view that mouse embryos begin to synthesize RA from retinol at approximately 7.5 dpc. Since our RA assay depended upon RA diffusion from the embryo to the reporter cells, it was impossible to determine in 7.5 dpc embryos, due to their small size, whether RA was released preferentially from the posterior end containing the primitive streak as suggested by these other studies (Rossant et al., 1991; Hogan et al., 1992). However, our analysis of older embryos (8.5–9.5 dpc) showed that RA release was indeed more concentrated in the trunk relative to the head. Our studies thus confirm these previous findings and provide a more direct assay for the presence of endogenous RA in mouse embryos.

The potential of various ADHs to function as RA synthetic enzymes during embryogenesis was examined by analyzing the spatiotemporal expression of their genes by whole-mount in situ hybridization. Expression of the class IV ADH gene followed the temporal pattern of RA detection, being observed at 7.5 dpc and thereafter but not at 6.5 dpc. Class IV ADH mRNA as well as RA were both observed in the embryonic tissues, but neither was observed in the extraembryonic tissues at 7.5 dpc. The RA reporter cell bioassay indicates that prior to 7.5 dpc...
there is an undetectable level of biologically active retinoids in mouse embryos, but by 7.5 dpc and thereafter the enzymatic machinery needed to convert retinol to RA has been established. This suggests that mouse class IV ADH, known to function as a retinol dehydrogenase in vitro (Connor and Smit, 1987), is expressed at the correct time to participate in the initiation of RA synthesis during gastrulation and may catalyze the oxidation of retinol to retinal, the first step of RA synthesis.

Class IV ADH gene expression was also detected in 8.5–9.5 dpc embryos. Prior to neural tube closure class IV ADH mRNA was transiently detected along the dorsolateral regions of the anterior neural folds and caudal neuropore. After neural tube closure class IV ADH mRNA was not detected in the neuroepithelium and was instead localized in the craniofacial region, forelimb buds, and posterior trunk. Our bioassay detected RA in most of the tissues expressing class IV ADH mRNA, particularly the craniofacial region, forelimb bud, and trunk (centered at the site of forelimb bud attachment). Thus, these tissues are apparently able to catalyze both steps of RA synthesis, retinol oxidation and retinal oxidation, with class IV ADH a very likely candidate for catalyzing the first step. We did not detect RA in the dorsolateral regions of the anterior neural folds in 8.5 dpc embryos subjected to the bioassay. However, this could be due to a threshold effect, since the class IV ADH expression in this tissue was transient and the neural folds were nearly completely fused after overnight incubation. Such embryos developed in vitro to approximately the 9.0 dpc stage, which, as indicated in our studies, has very little class IV ADH mRNA remaining in the neural folds.

Our bioassay detection of RA in the trunk of 8.5–9.5 dpc embryos, with a maximum in the prospective forelimb region, indicated an anterior skewing relative to the expression pattern of class IV ADH mRNA, which has a maximum just posterior to the prospective forelimb region. The RA indicator mice described above also indicate that the RA maximum in the trunk lies in the vicinity of the prospective forelimb, with less RA in the posterior trunk (Rossant et al., 1991; Balkan et al., 1992). This could indicate that the expression of class IV ADH sets up a field in which retinol is oxidized to retinal but that the synthesis of RA in that field is also dependent upon enzymes that can catalyze the oxidation of retinal to RA.
Class I ADH gene expression did not commence until 9.5 dpc and was limited to the mesonephros, a structure that gives rise to portions of the genitourinary system. Class I ADH functions as both a retinol dehydrogenase and an ethanol dehydrogenase in vitro (Boleda et al., 1993; Yang et al., 1994), but these results show that its gene is expressed too late to participate in embryonic RA synthesis during gastrulation. Despite this, class I ADH may contribute to the large amount of RA observed in the trunk of 9.5 dpc embryos. Also, class I ADH mRNA persisted in the mesonephros of 10.5 dpc embryos, whereas class IV ADH mRNA was absent in the embryo by this stage. Thus, class I ADH may contribute to the RA detected by others in the trunk of 10.5 dpc mouse embryos (McCaffery and Dräger, 1994). During late embryonic development and adulthood class I and class IV ADH mRNAs appear in the adrenal gland as well as the specialized epithelia of the genitourinary tract, respiratory tract, digestive tract, and skin, which are known to require RA for differentiation.

Class III ADH gene expression was already apparent by 6.5 dpc and continued nearly ubiquitously through 9.5 dpc. Expression of the class III ADH gene at the egg cylinder stage, which is prior to our observation of RA, suggests that its function is not to generate RA. In fact, class III ADH has been previously demonstrated to be inactive as a retinol dehydrogenase (Boleda et al., 1993; Yang et al., 1994), and instead has been shown to function as a glutathione-dependent formaldehyde dehydrogenase (Koivusalo et al., 1989). The ubiquitous production of mRNA for class III ADH is consistent with the proposed housekeeping role of this enzyme in removing metabolically generated formaldehyde and is inconsistent with a role in the synthesis of RA, which we have shown is present endogenously in a distinct spatial and temporal pattern.

A novel finding in our studies was the colocalization of endogenous RA and class IV ADH expression in the craniofacial tissues known to be populated primarily by migrating cranial
neural crest cells. Vital dye analysis of mouse embryos has shown that the dorsal neuroepithelia (i.e. dorsolateral regions of the neural folds) in the forebrain, midbrain, and hindbrain all contribute cranial neural crest cells, which exit the neuroepithelium and migrate ventrally during approximately 8.0–9.5 dpc to form the mesenchyme of the frontonasal mass, periorbital region, first branchial arch (maxillary and mandibular components), and the remaining branchial arches (Serbedzija et al., 1992; Osumi-Yamashita et al., 1994). A role for endogenous RA in the development of the craniofacial region is apparent from studies on mice mutant for both RARα and RARγ, which have malformations of almost all cranial neural crest mesenchymal derivatives (Lohnes et al., 1994). Also, a study using a lacZ transgene linked to an RA response element suggested that RA exists in the periorbital neural crest mesenchyme (Rossant et al., 1991; Balkan et al., 1992). Using a different method of detection, our studies indicate that endogenous RA does in fact exist in the craniofacial region from 9.0–9.5 dpc but is absent from the brain. In addition, we have found that class IV ADH expression at these stages occurs in the cranial neural crest and craniofacial region but not the brain. Our data thus provide evidence that after the initiation of RA synthesis in posterior tissues of primitive streak embryos, class IV ADH then participates in the initiation of RA synthesis anteriorly in the cranial neural crest. By 9.5 dpc, class IV ADH expression and significant RA levels were both associated with the craniofacial mesenchyme but not brain or neural tube tissue, suggesting that class IV ADH catalyzes RA synthesis in the cranial neural crest mesenchyme following the completion of neural crest cell migration from the neuroepithelium.

These findings have implications for the mechanism of ethanol-induced birth defects during human embryogenesis. Fetal alcohol syndrome is characterized by craniofacial defects of the eyes, upper lip, and jaw (short palpebral fissures, hypoplastic philtrum, maxillary hypoplasia, deformed palate, and micrognathia) arising from improper development of the cranial neural crest (Jones and Smith, 1973; Clarren and Smith, 1978). Very similar defects have also been observed in mouse embryos treated with ethanol during gastrulation (Sulik et al., 1981; Webster et al., 1983). Ethanol is a much poorer substrate for class IV ADH than retinol, but at very high doses ethanol has been shown to compete with retinol for access to the enzyme, leading to inhibition of retinol oxidation (Julià et al., 1986; Duester, 1995). We have proposed that the negative effects of excess ethanol consumption during pregnancy, as manifested by fetal alcohol syndrome, may be caused by an inhibition of ADH-catalyzed RA synthesis in the cranial neural crest leading to a failure of RAR function needed for normal development (Duester, 1991; Duester, 1994). In order for this hypothesis to be correct, an ADH capable of retinol oxidation must be expressed in the correct spatiotemporal pattern. Our observation of class IV ADH gene expression and endogenous RA in craniofacial tissues thus lends credence to this model.

A concentration gradient of endogenous RA has been proposed to play a role in anterior-posterior patterning of the embryo by differentially regulating members of the hox gene family (Simeone et al., 1990). This has been supported by

Fig. 4. Detection of class I and class III ADH mRNA in mouse embryos by whole-mount in situ hybridization. Class I ADH mRNA was not detected at 6.5 dpc (A), 7.5 dpc (B), or 8.5 dpc (C) but was detected at 9.5 dpc (D) in the mesonephros (m). Class III ADH mRNA was detected nearly ubiquitously at all stages analyzed, i.e. 6.5 dpc (E), 7.5 dpc (F), 8.5 dpc (G), and 9.5 dpc (H). Magnifications are as follows: ×63 (A–C and E–G), ×45 (H), and ×35 (D).
studies indicating a posterior preference for RA accumulation in embryos (Rossant et al., 1991; Balkan et al., 1992; Hogan et al., 1992; Chen et al., 1992; Chen et al., 1994; Wagner et al., 1992; McCaffery and Dräger, 1994; Horton and Maden, 1995). Our RA bioassay results support the existence of a posterior preference for RA accumulation in 8.5–9.5 dpc mouse embryos with the high point located in that region of the trunk containing the prospective forelimb buds and the low point located in the posterior midbrain-anterior hindbrain region. Class IV ADH expression followed this pattern as well, suggesting that this enzyme participates in establishing an anterior-posterior RA gradient along the embryo with the high end located in the trunk.

RA has also been found to exist in an anterior-posterior gradient in the chick and amphibian forelimb buds (Thaller and Eichele, 1987; Scadding and Maden, 1994), but no convincing evidence for such a gradient has been found in the mouse forelimb bud (Rossant et al., 1991; Scott et al., 1994). Our data also indicates that RA in the mouse forelimb bud is not localized in an anterior-posterior gradient. On the contrary, our analysis of 10.5 dpc mouse forelimb buds suggests that there may exist a proximal-distal gradient (high proximally). This finding is consistent with a study indicating that expression of a lacZ transgene linked to an RA response element is observed only at the proximal base of the forelimb bud where it attaches to the trunk (Rossant et al., 1991). Importantly, we have identified that class IV ADH expression is also preferentially localized in the proximal rather than distal forelimb bud mesenchyme, thus indicating that it may participate in RA synthesis in this location.

Neither class IV ADH nor class I ADH were expressed in the floor plate of the neural tube or the embryonic retina, suggesting that these retinol dehydrogenases do not participate in the synthesis of RA previously detected in those tissues (Wagner et al., 1990, 1992; McCaffery et al., 1993; McCaffery and Dräger, 1994). Since an aldehyde dehydrogenase that can oxidize retinal to retinol has been identified in these tissues (McCaffery et al., 1993; McCaffery and Dräger, 1994), further studies are needed to identify a retinol dehydrogenase responsible for the production of retinol.

The link we have found between class IV ADH gene expression and endogenous RA in mouse embryos has implications for our understanding of the mechanism of RA synthesis. Liver class I ADH was early established as a cytosolic NAD-linked retinol dehydrogenase that could catalyze the in vitro oxidation of retinol for RA synthesis (Bliss, 1951; Zachman and Olson, 1961). However, the role of this enzyme in vivo was questioned by the discovery of a class I ADH mutant mouse that could produce sufficient RA for survival (Posch et al., 1989). Subsequently, a liver microsomal NADP-linked retinol dehydrogenase (short chain dehydrogenase/reductase) that in vitro functions best with retinol bound to cellular retinol binding protein type I was proposed to be the physiological catalyst in the oxidation of retinol for RA synthesis (Posch et al., 1991; Chai et al., 1995). However, since this enzyme uses NADP(H) as its coenzyme it may preferentially bind NADPH (due to the low ratio of NADP to NADPH) and function as a retinal reductase to convert retinal to retinol. Cells normally maintain their NADP/NADPH ratio near 0.01, which favors metabolite reductase for enzymes using this coenzyme, while keeping their NAD/NADH ratio near 1000, which favors metabolite oxidation for enzymes using this coenzyme (Veech et al., 1969). These coenzyme concentrations would be expected to be important in directing the microsomal retinol dehydrogenase to function in the reductive direction and ADH retinol dehydrogenases in the oxidative direction. The reductive reaction is known to occur in the intestine where the breakdown of \( \beta \)-carotene (the ultimate source of all retinoids) produces retinal, which is reduced to retinol by a microsomal retinal reductase activity that functions best with retinal bound to cellular retinol binding protein type II (Kakkad and Ong, 1988). Conversion of \( \beta \)-carotene to retinal and retinol is also known to occur to a lesser extent in the liver and other organs (Olson, 1989). Thus, there exists a biochemical argument suggesting that the liver microsomal retinol dehydrogenase may not catalyze retinol oxidation in vivo but rather is involved in retinol reduction. The discovery that class IV ADH, originally isolated in the epidermis and stomach mucosa, functions as a NAD-linked retinol dehydrogenase (Connor and Smit, 1987; Boleda et al., 1993; Yang et al., 1994) opens the possibility that this form of ADH may be a physiological catalyst for RA synthesis. Class IV ADH is present in all mammals so far analyzed including the wild type and mutant deer mouse (Zhang et al., 1993; Zgombic-Knight et al., 1995). Thus, the absence of class I ADH in the mutant deer mouse may be compensated for by the presence of class IV ADH. We have now shown that the class IV ADH gene is expressed prior to the class I ADH gene during the onset of embryonic RA synthesis, suggesting that it plays a more crucial role in RA synthesis. Thus, our studies indicate that future investigations of the RA synthetic mechanism should put particular emphasis upon the role of class IV ADH. It is clear that we do not yet know the relative contributions of ADHs, microsomal retinol dehydrogenases, and cellular retinol binding proteins for RA synthesis during embryogenesis or subsequent maintenance of adult functions. Mutational studies of all these components may be necessary to determine what roles they play in RA synthesis or other aspects of retinoid metabolism.

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REFERENCES

Algar, E. M., Seeker, T. L., and Holmes, R. S. (1983) Eur. J. Biochem. 137, 139–147
Balkan, W., Colbert, M., Bock, C., and Linney, E. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3347–3351
Blaner, W. S., and Olson, J. A. (1994) The Retinoids: Biology, Chemistry, and Medicine, 2nd Ed. (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) pp. 229–255, Raven Press, Ltd., New York
Bliss, A. F. (1951) Arch. Biochem. 31, 197–204
Boleda, M. D., Saub, N., Ferrés, J., and Párs, X. (1993) Arch. Biochem. Biophys. 307, 85–90
Chai, X., Boerner, M. H. E. M., Zhai, Y., and Napoli, J. L. (1995) J. Biol. Chem. 270, 3000–3004
Chen, Y., Huang, L., Russo, A. F., and Solursh, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10056–10059
Choi, Y., Huang, L., and Solursh, M. (1994) Dev. Biol. 161, 70–76
Clarien, S. K., and Smith, D. W. (1978) N. Eng. J. Med. 296, 1063–1067
Connor, M. J., and Smit, M. H. (1987) Biochem. J. 244, 489–492
Daniellsson, O., Atiani, S., Luque, T., Hydén, N., González-Duarte, R., and Jörnvall, H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4980–4984
Dersch, H., and Zile, M. H. (1993) Dev. Biol. 160, 424–433
Duester, G. (1991) Alcohol Clin. Exp. Res. 15, 568–572
Duester, G. (1994) Vitamin A in Health and Disease (Blomhoff, R., ed) pp. 343–363, Marcel Dekker, Inc., New York
Duester, G. (1995) Drug Alcohol Abuse Rev. 6, 75–99
Hofmann, C., and Eichele, G. (1994) The Retinoids: Biology, Chemistry, and Medicine, 2nd Ed. (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) pp. 387–441, Raven Press, Ltd., New York
Hogan, B. L. M., Thaller, C., and Eichele, G. (1992) Nature 359, 237–241
Horton, C., and Maden, M. (1995) Dev. Dyn. 202, 312–323
Jones, K. L., and Smith, D. W. (1973) Lancet 2, 999–1001
Jullia, P., Ferrés, J., and Párs, X. (1986) Exp. Eye Res. 42, 305–314
Kakkar, R. B., and Ong, D. E. (1988) J. Biol. Chem. 263, 12911–12919
Kastner, P., Chambon, P., and Led, M. (1994) Vitamin A in Health and Disease (Blomhoff, R., ed) pp. 189–238, Marcel Dekker, Inc., New York
Kawamura, M. H. (1992) The Atlas of Mouse Development, Academic Press, Inc., San Diego
Kim, C.-I., Leo, M. A., and Lieber, C. S. (1992) Arch. Biochem. Biophys. 294, 388–393
Koivistoala, M., Baumann, M., and Uttila, L. (1989) FEBS Lett. 257, 105–109
Kim, L., and Chae, C. B. (1989) BioTechniques 7, 576–579
Löhne, D., Mark, M., Mendelsohn, C., Dahié, P., Dierich, A., Garry, P., Ganz, A., and Chambon, P. (1994) Development 120, 2723–2748
Maden, M. (1994) Vitamin A in Health and Disease (Blomhoff, R., ed) pp. 289–322.
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