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Dynamic Patterns of Retinoic Acid Synthesis and Response in the Developing Mammalian Heart

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Retinoic acid (RA) has been implicated in cardiac morphogenesis by its teratogenic effects on the heart, although its role in normal cardiogenesis remains unknown. To define the parameters of RA action in cardiac morphogenesis, we analyzed the patterns of ligand synthesis, response, and inactivation in the developing mouse heart. Activation of a lacZ transgene controlled by an RA response element (RARE) was compared to the localization of the retinaldehyde-oxidizing dehydrogenase RALDH2, the earliest RA synthetic enzyme in the mouse embryo, and to the expression of a gene encoding an RA-degrading enzyme (P450RA). We observed that RALDH2 localization and RA response were virtually superimposable throughout heart development. Initially, both RALDH2 and RARE-LacZ activity were restricted to the sinus venosa in unlooped hearts, but were high in the dorsal mesocardium, while P450RA expression was restricted to the endocardium. Later stages were characterized by a sequential, noncontiguous progression of RALDH2 accumulation and RA response, from the sinus venosa to atria, dorsal–medial conotruncus, aortic arches, and the epicardium. This dynamic pattern of RA response was a direct result of localized RALDH2, since hearts of cultured embryos were uniformly competent to respond to an exogenous RA challenge. These observations support a model in which the influence of endogenous RA on heart development depends upon localized presentation of the ligand, with only limited diffusion from the source of its synthesis.

INTRODUCTION

Retinoids comprise a group of regulatory molecules implicated in numerous biological functions in embryogenesis, from early patterning of the body axes to morphology of organ formation (Conlon, 1995; Smith and Dickman, 1997). The heart is one of the most sensitive organs to perturbations of retinoid concentration throughout its development (Kastner *et al.*, 1994). The profound influence of retinoids on vertebrate heart development is illustrated by the numerous cardiac malformations induced by exposure to excess retinoids during pregnancy, including aortic and cardiac defects such as transposition of the great arteries, septal defects, and

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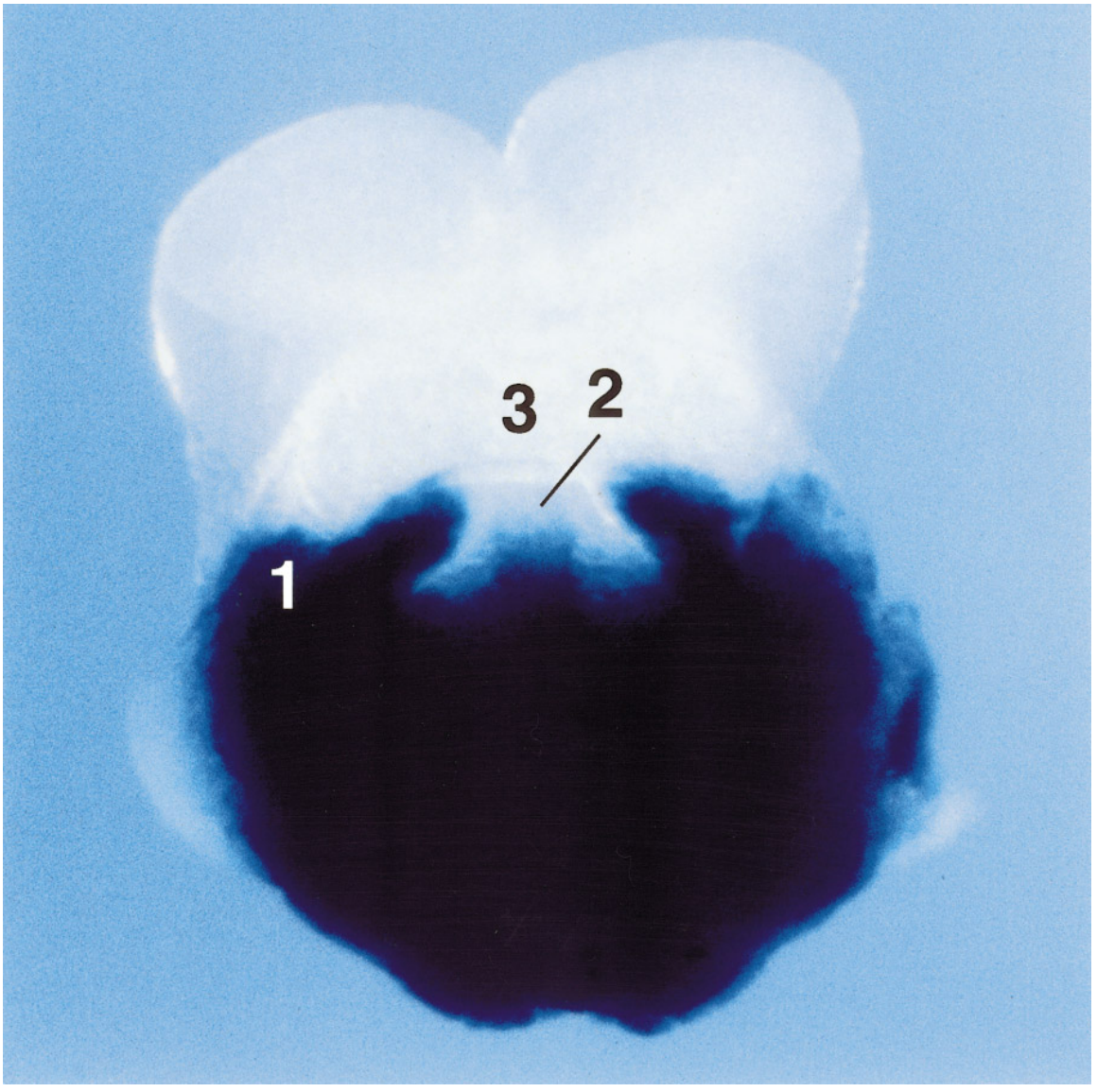


FIG. 1. RA response is highly restricted in early heart development. Ventral view of an 8.25-dpc RARE-lacZ transgenic mouse embryo (4–5 somite pairs). At this stage of development, β-gal staining is observed over trunk areas extending to the caudal neuropore (not shown). A sharp anterior limit of transgene expression includes the prospective sinus venosa (1) and the anterior intestinal portal (2). Note that transgene expression is completely absent from anterior portions of the heart (3).

reversion of heart laterality (Pexieder *et al.*, 1995; Dickman and Smith, 1996). Insufficient nutritional intake of retinoids can also lead to an overlapping set of defects, suggesting an important morphogenetic role for these molecules in heart development (Twal *et al.*, 1995).

Investigation of the mechanisms whereby retinoids affect developmental processes have focused largely on exposure to exogenous retinoic acid (RA), on vitamin A deprivation, and on the inactivation of RA effector molecules by gene targeting. The dramatic teratogenicity of retinoid excess or deficiency in vertebrate embryogenesis underscores the importance of regulating local concentrations of these powerful morphogens. The significance of RA-induced teratogenesis is limited, however, since the effects of exogenous RA do not necessarily elucidate the physiological role of endogenous RA during

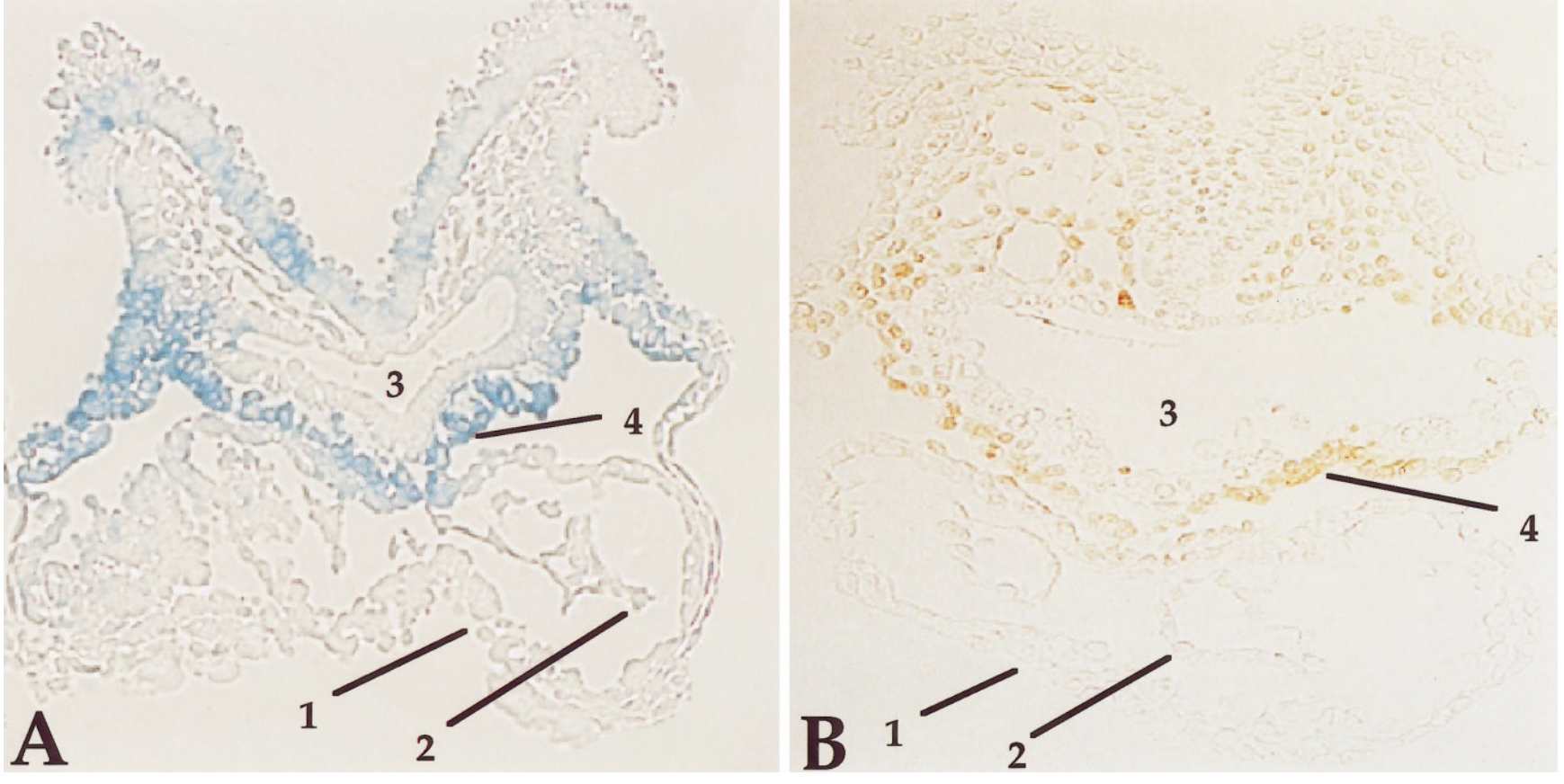


FIG. 2. RALDH2 enzyme and RA response overlap in extracardiac tissues. (A) β-Gal activity visualized in a transverse section from an 8.5-dpc transgenic embryo is absent within the myocardium (1), the endocardium (2), and foregut epithelium (3) but present in the dorsal mesocardium (4). (B) A transverse section from an 8.5-dpc embryo treated with an anti-RALDH2 antibody. RALDH2 expression is also absent from the myocardium (1), the endocardium (2), and the foregut epithelium (3) and present in the dorsal mesocardium (4), illustrating the superimposition of domains of RALDH2 expression and response at this stage.

development. Likewise, the effects of vitamin A deficiency on embryogenesis are thought to reflect critical requirements for RA (Dickman *et al.*, 1997; Smith and Dickman, 1997). Yet, vitamin A deprivation during development can only reveal the consequences of a protracted RA-deficient state and it is clear that this model cannot provide information on the precise cellular and temporal requirements for RA (Dickman *et al.*, 1997).

RA and its derivatives exert their influence on transcriptional control of gene expression through their effectors, the RAR and RXR families of nuclear receptors (Mangelsdorf and Evans, 1995). The distribution of RA effectors in the embryo yields few clues to the specificity of their action. *In situ* hybridization studies show that RARs and RXRs are widely expressed throughout the embryo, including the forming heart (Ruberte *et al.*, 1991; Mangelsdorf *et al.*, 1992; Dolle *et al.*, 1994). Yet, congenital abnormalities in mice homozygous for null mutations in the genes of single receptors are restricted to only a few sites expressing those receptors during embryogenesis. The general lack of correspondence between receptor transcript abundance and a particular defect make it difficult to assign specific roles to any one receptor in heart development (Smith and Dickman, 1997). Recent studies, however, reveal that combined ablation of RXRα and the RAR genes has a disproportionate effect on RA signaling. RXRα/RARβ double mutants are defective in conotruncal septation while in RXRα/RARα double mutants the cephalic arteries and articopulmonary septa are inappropriately patterned (Sucov *et al.*, 1994; Kastner *et al.*, 1997a). This suggests that RXRα/RAR heterodimers mediate proper heart development.

Endogenous retinoids are produced from conversion of dietary vitamin A to retinol, which can be successively oxidized to produce retinaldehyde and various forms of RA (Means and Gudas, 1995). In addition, retinol can be metabolized to other active ligands with diverse effects, including 4-oxoretinol, which induces growth inhibition of tumor cell lines (Guo and Gudas, 1998). The enzymes that mediate these essential synthetic pathways have not been completely characterized biochemically, and the importance of retinoid synthetic pathways as a potential control point in cardiac morphogenesis is as yet unkown. In the vertebrate embryo, RA is present in the primitive streak stage and may play a role in cardiac morphogenesis prior to heart tube formation (Hogan *et al.*, 1992). Current evidence implicates the recently characterized RALDH2 as a key enzyme catalyzing the dehydrogenation of retinaldehyde to RA (Zhao *et al.*, 1996) at these early stages of development (7.0–7.5 dpc). RALDH2 belongs to a group of NAD-dependent aldehyde dehydrogenases that are present in early development, as opposed to FAD-coupled aldehyde and xanthine oxidases, which are activated at later stages. RALDH2 is initially responsible for the generation of endogenous RA in early embryogenesis (7.0–8.5 dpc) at a time when other RA synthetic enzymes have not yet been activated, and it continues to be the most abundant retinaldehyde dehydrogenase in the trunk area throughout development (Niederreither *et al.*, 1997). Together with the recently described V1 retinaldehyde dehydrogenase activity (McCaffery and Drager, 1995), RALDH2 shows more specificity for retinaldehyde substrate than does the previously described cytosolic class 1 aldehyde dehydrogenase, ALHD2. At early developmental stages, both the V1 enzyme and ALHD2 are mainly expressed in the eye and face region, while RALDH2 is responsible for most of the biosynthesis of RA in areas caudal to the head.

A necessary prerequisite for defining the role of RA as a morphogen during cardiogenesis is the localization of endogenous ligand in the developing heart and the definition of the resulting regions of RA response. Previous studies of the whole embryo have demonstrated regional differences in RA levels (Hogan *et al.*, 1992), and in the expression of the RALDH2 transcript during mouse embryogenesis (Niederreither *et al.*, 1997), but neither the distribution of RA nor its synthetic and inactivating enzymes have been described in the heart. To ascertain whether cardiac patterning is influenced by RA availability or by differential sensitivity to RA stimulation, we conducted a detailed analysis of RA responsiveness and RALDH2 distribution during mouse heart development (8.25–13.5 dpc). We took advantage of a previously characterized RA-responsive lacZ transgene (Rossant *et al.*, 1991) to monitor the response to endogenous RA. We also analyzed the expression pattern of a gene encoding a cytochrome P450 RA-inactivating enzyme, P450RA (Fujii *et al.*, 1997). Our study revealed a dynamic pattern of RALDH2 expression and RA response in the developing mouse heart, despite the uniform competence of the embryonic heart to respond to RA in culture. These results indicate that RA action during this critical period of heart development is mediated by localized ligand synthesis.

MATERIALS AND METHODS

Transgenic Mice

Transgenic mice have been previously described (Rossant *et al.*, 1991) in which the bacterial lacZ gene coding for β-galactosidase (β-gal) is driven by a heat shock promoter fused to RA-responsive elements from the RARβ promoter. Litters heterozygous for the transgene were obtained by breeding a homozygous male with wild-type CD1 females. Noon on the day of vaginal plug was designated as 0.5 dpc and developmental stages were confirmed by their morphology and somite number. For histological analysis, at least 10 embryos were compared at all stages.

Xgal, Antibody, and in Situ Hybridization Staining

Embryos and hearts spanning the stages 8.25 through 13.5 dpc were collected and analyzed for β-gal activity, RALDH2 protein, or P450RA transcripts. β-Gal activity was detected using Xgal as substrate and embryos were treated as described (Cheng *et al.*, 1993). For immunohistochemical analysis (Dent *et al.*, 1989), a rabbit polyclonal RALDH2 antibody was used at a dilution of 1:200. The secondary antibody was a horseradish peroxidase-labeled donkey anti-rabbit IgG (Amersham) used at a 1:1000 dilution. Whole mount RNA *in situ* hybridization was performed using a P450RA cDNA clone (No. 10245), a generous gift of Dr. H. Hamada (Fujii *et al.*, 1997). Antisense and sense probes were produced with T3 and T7 RNA polymerase, respectively. Embryos were hybridized using a revised protocol (Wilkinson, 1992). The sense probe gave no detectable staining (not shown).

Embryo sections were prepared by dehydrating embryos through an ethanol series to 95% ethanol:5% phosphate-buffered saline. After 1 h at room temperature in JB4 infiltration resin (Polysciences), embryos were transferred to molding trays, all liquid was removed, and fresh, catalyzed JB4 resin was added. Polymerized blocks were cut using a glass knife on a Leica No. 2065 microtome. The resulting 5 μM serial sections were mounted onto Colorfrost Plus slides (Fisher).

Paraffin sections were generated by standard methods (Sassoon and Rosenthal, 1993) and treated with 1:400 dilutions of rabbit polyclonal RALDH2 antibody and of a monoclonal antibody against β-gal (JIE7, DSHB) followed by a 1:800 dilution of anti-rabbit and anti-mouse IgG–biotin conjugate (B7389 and B7151, Sigma) and by a 1:200 dilution of an avidin–alkaline phosphatase conjugate (E2636, Sigma). The chromogenic substrate Fast Red (Sigma) was used to identify regions of RALDH2 expression and BCIP/NBT to identify regions of β-gal activity.

Embryo Cultures

Staged 8.5 dpc embryos were dissected in phosphate-buffered medium (Cockcroft, 1990) at room temperature, carefully removing Reichert's membrane. Embryos with beating hearts were cultured in 75% rat serum:25% Dulbecco's modified Eagle medium. Serum was prepared as previously described (Cockcroft, 1990) from blood obtained via the renal artery of CD1 male rats. All-*trans*- or 9-*cis*-retinoic acid (Sigma) was dissolved in DMSO to 10^{-3} M. One microliter of RA (to a final concentration of 10^{-6} M) or 1 μL of DMSO alone (untreated) was added to 1 mL of serum. Those embryos with hearts still beating after 24 h were washed with PBS and prepared for Xgal staining, immunohistochemistry, or whole mount *in situ* hybridization.

Image Analysis and Processing

Fixed embryos were visualized on a Leica MZ8 dissecting microscope and photographed with 160 ASA tungsten slide film. Histological sections were photographed on a Zeiss Axiophot microscope using 100 ASA slide film. Digital images were generated by scanning slides on a Polaroid sprint scan 35 film scanner.

RESULTS

Retinoic Acid Synthesis and Response Are Highly Restricted within the Fused Heart Tube

To characterize the patterns of RA response in the developing mouse heart, we monitored expression patterns of a lacZ transgene driven by the RA response element from the RARβ promoter (Rossant *et al.*, 1991). LacZ gene expression was extensive in caudal structures of 8.25-dpc whole mount mouse embryos (Fig. 1) but was restricted in the heart to the sinus venosa, the paired venous inflow tracts located at the caudal end of the recently fused heart tube. We observed no RA responsiveness in anterior regions of the heart at this stage.

In transverse sections of 8.25-dpc embryos, RA response (Fig. 2A) was localized to the neurectoderm of the unfused neural tube and to some mesodermal structures, including the dorsal aspect of the pericardium and the dorsal mesocardium. Myocardial and endocardial tissues were negative at this stage, as was the foregut endoderm.

Retinoic Acid Response Colocalizes with the Expression of Its Synthetic Enzyme

We hypothesized that patterns of RA response could be linked to localized RALDH2. As monitored with a polyclonal antibody to the RALDH2 enzyme in transverse sections (Fig. 2B), RALDH2 was present in the dorsal mesocardium, while the anterior portion of the fused heart showed no enzyme at this stage. Synthetic activity was also absent in the foregut endoderm, indicating that the activation of RA-responsive genes in the embryo correlates well with localized expression of RA synthetic enzyme.

We further examined the pattern of RALDH2 expression and RA response in embryos collected at 8.5 dpc, when the heart tube had looped (Fig. 3). Transgenic embryos were stained for lacZ expression to visualize RA responsiveness (Fig. 3A), while RALDH2 was monitored using the RALDH2 antibody in whole mount embryos (Fig. 3B).

Although RA response in axial structures of the embryo extended rostrally past the heart at this time (Fig. 3A), it did not extend ventrally into the heart beyond the sinus venosa and atria, with the exception of a small region in the dorsomedial conotruncus (not shown). Cranially, eye tissue was RA responsive, while the tail region was essentially negative, except for two symmetrical areas at the base of the allantois which express RALDH2 mRNA (Niederreither *et al.*, 1997) and RALDH2 enzyme (not shown). This pattern of ligand response correlated well with that of RALDH2 expression, since immunohistochemical analysis of RALDH2 enzyme generated a similar expression pattern (Fig. 3B). In the eye region,

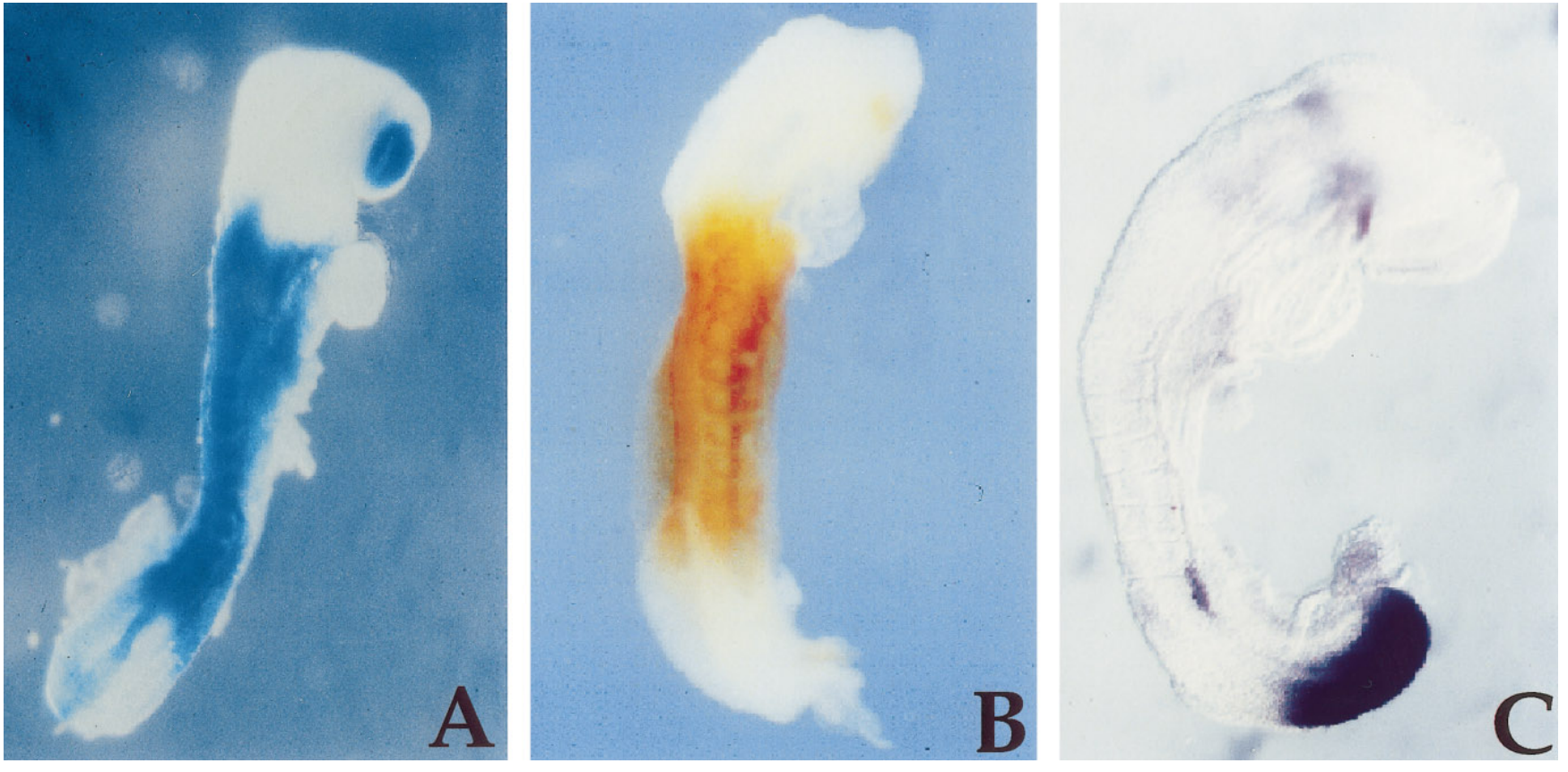


FIG. 3. Patterns of RALDH2 distribution and RA response but not inactivation are superimposible in the mouse embryo. Right lateral views of 8.5-dpc RARE-lacZ transgenic embryos (7–8 somite pairs) submitted to staining for β-gal activity (A), immunohistochemistry with an anti-RALDH2 polyclonal antibody (B), and *in situ* hybridization with a P450RA antisense riboprobe (C). At this stage of development, β-gal activity persists in the trunk and is activated in the eye region, but has receded from the caudal neuropore. RALDH2 enzyme overlaps areas of transgene activation, although the domains of expression are slightly broader than the domains of RALDH2. P450RA expression in the cephalic domain includes prospective rhombomere 2, epithelium of the foregut, and the first branchial arch. The caudal neuropore is intensely labeled. The sinus venosa and dorsal aorta as well as the prospective forelimb bud are positive for P450RA.

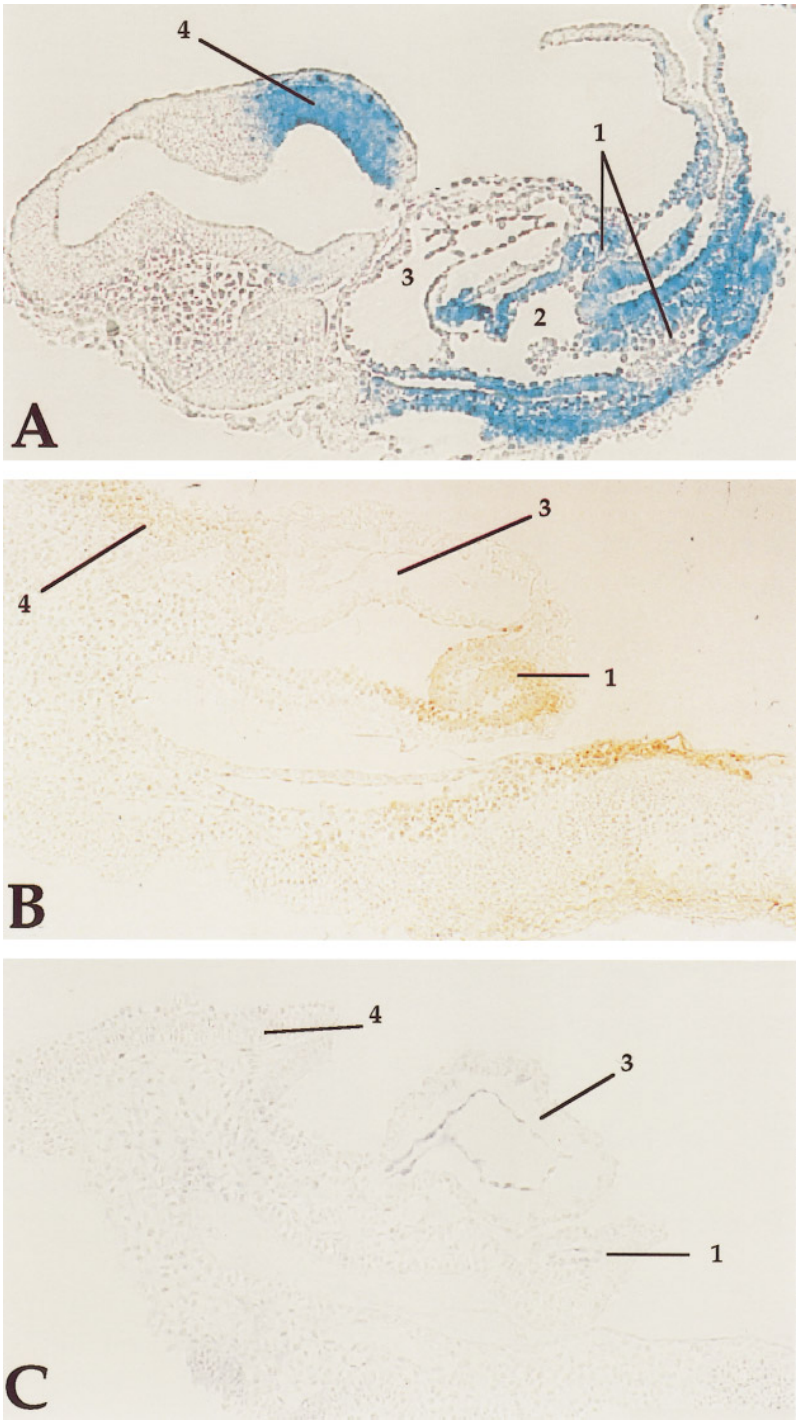


FIG. 4. RA synthetic enzyme and RA response colocalize in the heart while RA-inactivating enzyme is restricted to the endocardium. Sagittal sections of 8.5-dpc RARE-lacZ transgenic embryos were stained for β-gal (A), treated with the RALDH2 antibody (B), or treated with the P450RA antisense riboprobe (C). RA response (A) and synthesis (B) colocalize within the sinus venosa (1) and atrium (2), as well as the eye (4). The ventricle does not synthesize or respond to RA (3). P450RA expression (C) is restricted to the endocardial cells of the sinus venosa (1) and ventricle (3).

enhanced lacZ expression may reflect additional RA synthesis by the V1 retinaldehyde dehydrogenase (McCaffery and Drager, 1995).

Distinct Expression Patterns of RALDH2 Synthetic and Inactivating Enzymes

To further investigate possible control mechanisms of RA regionalization in the embryo, we examined the pattern of an RA-inactivating enzyme recently identified in the mouse by Hamada and colleagues (Fujii *et al.*, 1997), visualized with an antisense riboprobe (P450RA, Fig. 3C). The RA-inactivating enzyme was expressed at very high levels in the tail region (Fig. 3C), an area low in RALDH2 enzyme and RA response. Other regions of P450RA expression included the hindbrain and cranial neural crest, as well as a discrete band in the lateral plate mesoderm in a region underlying the developing forelimb bud (Fig. 3C).

Parasagittal sections were prepared from 8.5- to 8.75-dpc embryos and analyzed for lacZ activity, RALDH2 enzyme, and P450RA transcripts. RA response was found in the eye, the common atrium, and the sinus venosa (Fig. 4A), but not in the ventricle. RALDH2 was also expressed in the walls of the sinus venosa (Fig. 4B) and atrium (Fig. 6). Transcripts encoding P450RA were found in endocardial cells but not in myocardial cells of the sinus venosa and ventricle (Fig. 4C), indicating that RA-synthetic and -inactivating enzymes were localized in distinct patterns at this stage of heart development.

The Embryonic Heart Is Uniformly Competent to Respond to All-trans-Retinoic Acid

Although the RARE-lacZ transgene was activated in a very limited pattern at a critical stage in the development of the mouse heart (8.5 dpc), it was not clear if this pattern reflected RA availability, or a differential sensitivity to a more widespread RA stimulation. Although all-*trans*-RA is the predominant retinoid ligand in the early embryo, other forms such as 9-*cis*-RA have been detected at later stages. However, current chromatographic methods are relatively insensitive to adequately quantitate 9-*cis*-RA (Dräger and McCaffery, 1997). Therefore, to address if transgene activation is a function of all-*trans*- and 9-*cis*-RA availability, or differential sensitivity to stimulation by these retinoids, we cultured 8.5-dpc transgenic embryos with 1 μM all-*trans*- or 9-*cis*-RA (Fig. 5), monitoring RA response, synthesis and degradation. As seen in Fig. 5A, all cells in the 8.5-dpc embryo were competent to respond to all-*trans*-RA stimulation as previously described (Rossant *et al.*, 1991). In contrast, treatment with 9-*cis*-RA expanded the RA response domains cranially, ventrally into the heart, and caudally into the neuropore, but did not reproduce the widespread activation seen for all-*trans*-RA. RALDH2 expression was relatively insensitive to exogenous RA (Fig. 5B).

Analysis of the P450RA transcripts after treatment with either of the two ligands (Fig. 5C) revealed patterns similar but not identical to those previously described (Fujii *et al.*, 1997). The cranial domain of P450RA expression was upregulated and the caudal domain was downregulated by addition of either ligand. Both all-*trans*- and 9-*cis*-RA caused a rostral extension of expression of the caudal domain in a thin stripe along the dorsal aspect of the embryo. These results suggested that RA availability was the critical step in the activation of transgene expression in the developing heart.

A Dynamic Pattern of Retinoic Acid Synthesis and Response during Heart Development

To explore the patterns of RALDH2 expression and RA response in cardiac tissues, we compared the progression of RA-induced gene expression with the pattern of RALDH2 immunoreactivity at later stages of heart development. Figure 6 depicts ventral views of RA response and synthesis in embryonic hearts dissected at 1-day intervals from 8.5 to 13.5 dpc. The unlooped heart was initially devoid of response in all but the sinus venosa (Fig. 1, 8.25 dpc). Subsequently, progression of RA-induced responsiveness was rapid (Fig. 6, 8.5 dpc), showing lacZ staining in the sinus venosa, common atrium, and in the dorsomedial wall of the outflow tract. As development proceeded (Fig. 6; 9.5 dpc), RA responsiveness persisted in the sinus venosa, common atrium, and the dorsomedial wall of the conotruncus, while RALDH2 immunoreactivity was only seen in the sinus venosa and the common atrium.

At 10.5 dpc RA responsiveness was seen throughout the atria, in the dorsal aspect of the conotruncus, and in a few cells in the interventricular sulcus, but was largely absent from the ventricles. RALDH2 immunoreactivity was also observed in the atria and over the interventricular sulcus. One discrepancy between RALDH2 enzyme localization and response was apparent within the dorsomedial wall of the conotruncal region as early as 8.5 dpc, but was more pronounced at 10.5 dpc. Subsequently, RA response at 11.5 dpc was greatly reduced, with some residual β-gal activity still present along the dorsomedial wall of the conotruncus. At 11.5 dpc, RALDH2 immunoreactivity had decreased in the atria, while the ventricles showed a low level of staining. At 12.5 dpc, whole heart staining revealed a major increase in RALDH2 enzyme distribution. RA responsiveness was intense over the ventricle, with patchy staining in the atria. By 13.5 dpc, RA response had decreased while RALDH2 synthesis was sustained.

To delineate in detail the patterns of RALDH2 expression

and RA responsiveness at later stages of heart development, when the larger size of the organ resists complete penetration of substrate or antibodies, paraffin sections of 12.5-dpc transgenic hearts were prepared and analyzed for RALDH2 and lacZ gene products (Fig. 7). Only the ventricular epicardium, but not the underlying ventricular myocardium, showed RALDH2 immunoreactivity. Nevertheless, lacZ gene expression extended throughout the myocardial wall and trabeculae while the cushions did not appear to be responsive to RA at this stage.

DISCUSSION

Embryologists have long appreciated the importance of morphogen localization in establishing regional identity during development. Restricted production of a ligand with limited diffusion potential may be a key feature of its function. Nevertheless, relatively little attention has been paid to the synthetic and inactivating pathways leading to local differences in retinoid concentration, although it would seem particularly important in the case of morphogens such as RA, which are difficult to detect directly.

In this study we examined the correspondence of RA synthetic activity and RA response during heart development in the mouse. We observed a striking correlation between the domains of RA response and immunoreactivity to a retinaldehyde dehydrogenase (RALDH2) that catalyzes the last step in the RA biosynthetic pathway. These results support a major role for RALDH2 in the control of RA metabolism in the developing heart and indicate that morphogenetic actions of RA in the heart are effected through dynamic changes in the localization of its synthetic enzyme, rather than by diffusion or by changes in patterns of RA inactivation.

RALDH2 Is an Indicator of Initial RA Synthesis in the Embryo

RA metabolism is a multistep process involving different enzymes that metabolize retinol to RA (Means and Gudas, 1995). The first reaction in this oxidative process involves the oxidation of retinol to retinaldehyde by alcohol dehydrogenases such as ADH IV (Ang *et al.*, 1996). RA, the functional ligand which binds receptors, is a product of the second step, the dehydrogenation of retinaldehyde by aldehyde dehydrogenases such as RALDH2, suggesting that conversion of retinaldehyde to RA, rather than the dehydrogenation of retinol to retinaldehyde, represents the critical step in the control of RA biosynthesis. Although transcripts encoding alcohol dehydrogenases localize within the domain of RA responsiveness (Ang *et al.*, 1996; Napoli, 1996), RALDH2 expression more closely reproduces the RA response pattern (Figs. 2–4, 6). For example, at 8.5 dpc, ADH IV mRNA is distributed along the neural folds, trunk, and caudal neuropore of the mouse (Ang *et al.*, 1996), while both RALDH2 immunoreactivity and RA response are already restricted to the trunk regions, and are absent from the caudal neuropore (Fig. 3).

RALDH2 also appears to play a major role during early heart development. The analysis in Fig. 6 verifies that patterns of RALDH2 expression are almost superimposable to the regions of endogenous RA responsiveness. These sharply defined patterns are summarized in diagrammatic form in Fig. 8 and indicate that diffusion of RA, once synthesized, must be minimal, since the heart is uniformly competent to respond to RA in cultured embryos at 8.5 dpc (Fig. 5).

Implications of RALDH2 Expression and RA Response in Heart Development

The association between RA response and posterior regions of the developing heart suggests a model for the specification of cardiac inflow areas. RALDH2 is expressed at late primitive streak stages (7.0–7.5 dpc; Niederreither *et al.*, 1997). At this time, most cardiac precursors have already involuted into the primitive streak and have initiated their anterior-lateral migration (Tam *et al.*, 1997). Therefore, only the latest cardiac precursors, the cells that are fated to give rise to the sinus venosa and common atrium, are likely to express RALDH2 in the primitive streak. Thus, it is likely that the ability to synthesize RA is an important factor in the specification of inflow structures of the heart.

An association between RA signaling and the establishment of posterior heart regions during early development is supported by studies of vitamin A deprivation of quail embryos (Heine *et al.*, 1985; Dersh and Zile, 1993) as well as administration of a monoclonal antibody against all-*trans*-RA (Twal *et al.*, 1995). Anteriorizing defects included the formation of a single closed ventricle and the absence of vessels such as cardinal veins and omphalomesenteric veins that connect the embryonic and extraembryonic circulation to the inflow area of the heart, respectively. Alternatively, retinoid excess posteriorizes the heart. In zebrafish embryos, administration of RA causes deletion of anterior (ventricular) structures (Stainier and Fishman, 1992), whereas in the chick, excess RA causes the domains of atrial myosin heavy chain (MHC1) expression to expand anteriorly, at the expense of ventricular MHC1 expression (Yutzey *et al.*, 1994). The initial restriction of RALDH2 to posterior regions in the developing mouse heart, as revealed in the present study, provides additional support for the role of RA in the specification of cardiac inflow structures.

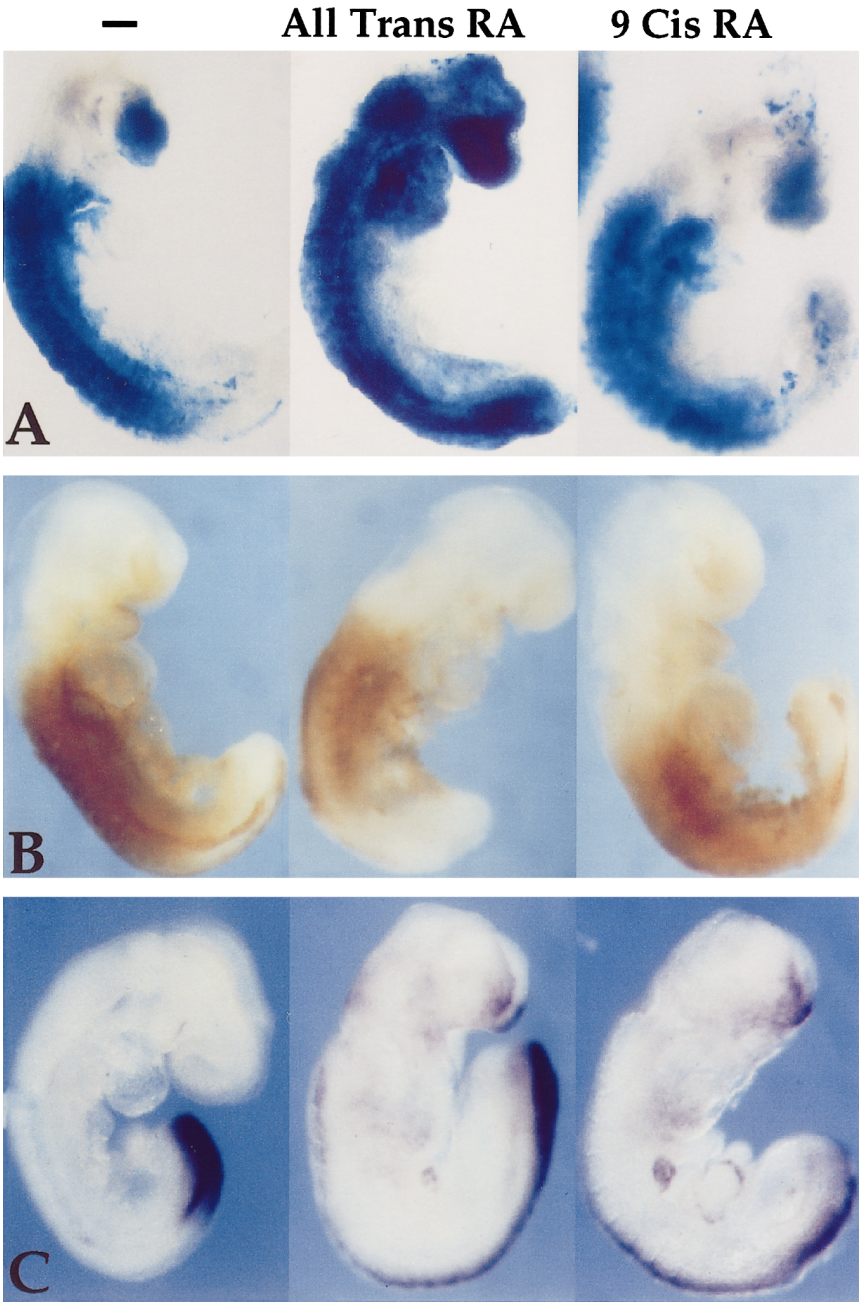
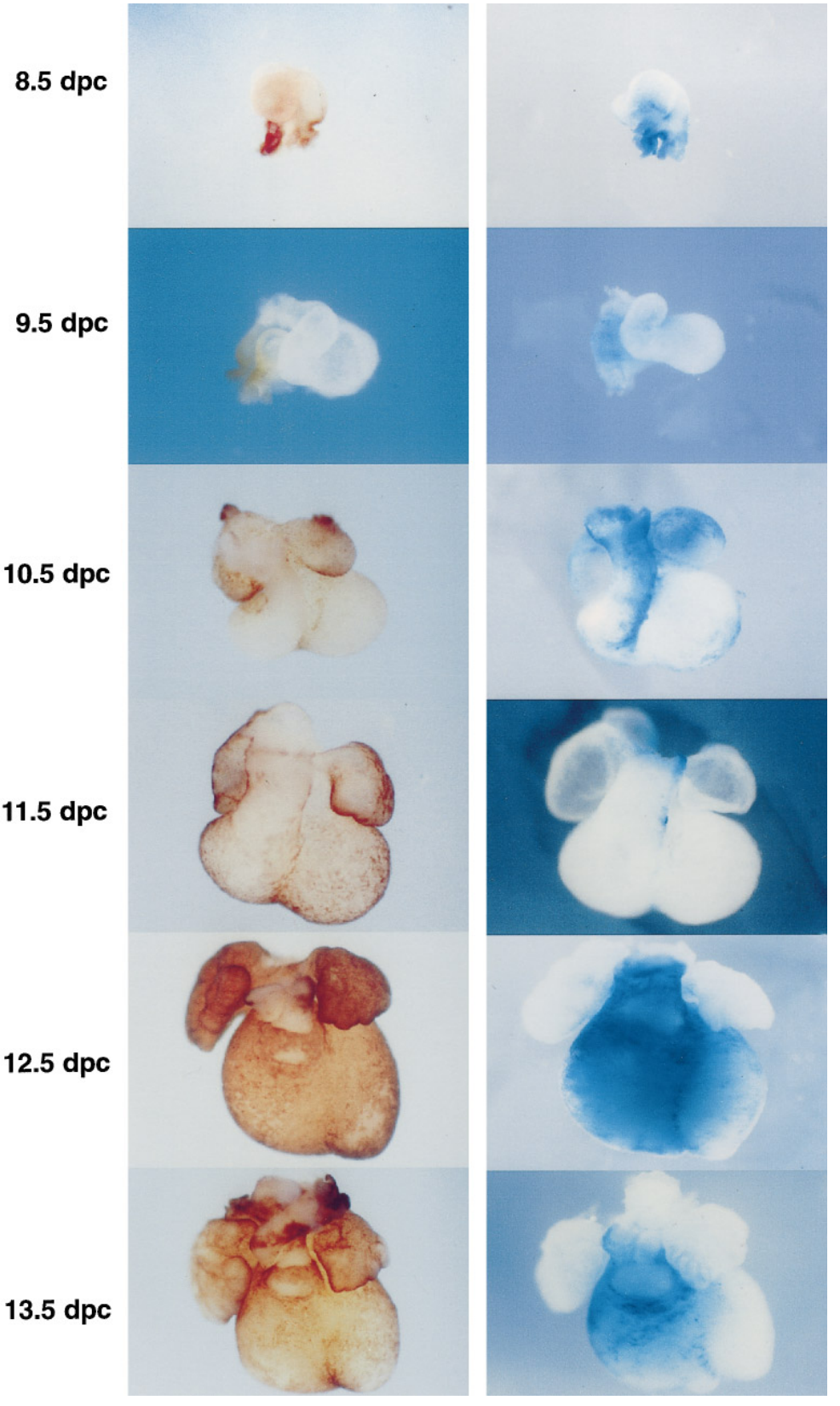


FIG. 5. The mouse heart is competent to respond to RA at 8.5 dpc. Dissected RARE-lacZ embryos with beating hearts were cultured in 75% rat serum/25% Dulbecco's modified Eagle medium in the absence or presence of all-*trans*- or 9-*cis*-retinoic acid. Those embryos with hearts still beating after 24 h were washed with PBS and stained with Xgal (A), RALDH2 immunohistochemistry (B), or a P450RA antisense probe (C). (A) Treatment with all-*trans*-RA induced β-gal activity throughout the whole embryo, including the heart. Treatment with 9-*cis*-RA, however, produced only patchy activation of β-gal activity in the head region, heart, and caudal neuropore. (B) Treatment with all-*trans*-RA induced a slight downregulation of RALDH2 immunoreactivity in trunk and caudal areas, whereas treatment with 9-*cis*-RA did not. (C) P450RA expression was also influenced by treatment with RA. The cephalic domain of expression was expanded while expression in the caudal domain was reduced posteriorly and expanded anteriorly in a thin, dorsal stripe. P450RA expression was equally affected by either all-*trans*- or 9-*cis*-RA.



Morphogenetic Fields of RA Signaling during Cardiac Development

The dynamic progression of RA signaling during heart development occurs in two waves initiated at the time of fusion of the heart primordia (Fig. 1), when RA response is first apparent in the sinus venosa. Shortly after looping, RA response appears over the dorsomedial surface of the conotruncus, while excluding the ventricles. By 11.5 dpc, RALDH2 expression and RA response are dramatically diminished throughout the heart, although RA response in aortic arches is high at this time (data not shown), suggesting that RA signaling in the developing great vessels is controlled by a largely independent program. At 12.5 dpc, a surge in RALDH2 occurs in the atrial and ventricular epicardium and in RA response within the epicardium and myocardium. Thereafter, RA response decreases steadily, persisting in limited areas of the ventricle as late as 1 day after birth (data not shown).

These patterns of RALDH2 localization and RA response preclude a simple caudal-to-rostral progression of RA action in the linear heart tube of the mouse. Rather, they represent three different morphogenetic fields controlled by RA during heart development. Posterior regions of the heart, the inflow tract, represent the first morphogenetic field. The second field is constituted by the dorsomedial wall of the conotruncus, destined to form nonmyogenic components of the mature heart, such as the aorta, pulmonary arteries, and semilunar valves. In this field, RA may be synthesized by a cranial neural crest cell-associated retinaldehyde dehydrogenase other than RALDH2 (Ang *et al.*, 1996). The third field is represented by the ventricles, which until 12.5 dpc are almost excluded from RA signaling. The striking contrast between the low levels of RA metabolism in the heart at 11.5 dpc and its dramatic increase within the ventricular epicardium at 12.5 dpc suggests that the timing of exposure to RA is critical for the proper development and expansion of the ventricles.

Discrepancies between RALDH2 Distribution and RA Response

During heart development, a lack of correspondence between RALDH2 localization and RA response was noted between 8.5 and 10.5 dpc, when lacZ staining appeared in the dorsomedial wall of the conotruncus where RALDH2 immunoreactivity was absent. Additionally, RALDH2 enzyme expression was considerably higher than RA response in the atria from 12.5 to 13.5 dpc (Fig. 3). Finally, RALDH2 enzyme was present exclusively in the ventricular epicardium at this stage, whereas RA response extended throughout the myocardial layers of the ventricle.

These discrepancies may reflect different biological phenomena. At 8.5–10.5 dpc, RA response in the dorsal conotruncus could be due to limited diffusion of RA from the adjoining atria, which express abundant levels of RALDH2 enzyme at this time (Fig. 6). Alternatively, RA response in the conotruncus, an area populated by cranial neural crest cells, may reflect RA synthesis by an enzyme other than RALDH2. Neural crest cell-derived structures are established targets for the teratogenic actions of RA excess and deficiency and express RA biosynthetic enzymes such as ADH IV. Therefore, it is likely that expression of ADH IV in these cells represents a source of RA generated by a retinaldehyde dehydrogenase other than RALDH2. This possibility is supported by studies showing accumulation of labeled RA and expression of the retinoic acid binding protein CRABP in cranial neural crest cells (Denker *et al.*, 1990).

The attenuation of RA response in the atria between 12.5 and 13.5 dpc could be due to several possible mechanisms. Although RALDH2 immunoreactivity is clearly present, RA levels can be downregulated either by changes in expression of genes in the RA biosynthetic pathway, or by posttranslational modifications of the enzyme. Alternatively, the atria may abrogate response to locally synthesized ligand by its inactivation or by downregulating the components of the RA-signaling

FIG. 6. Time course of retinoic acid synthesis and responsiveness during cardiac development in the mouse. Ventral and lateral views of hearts from 8.5- to 13.5-dpc embryos indicate colocalization of RALDH2 (left) and RA responsiveness (right). 8.5 dpc, RALDH2 immunoreactivity and β-gal activity can be seen in the sinus venosa and in the primitive ventricle. 9.5 dpc, right lateral view shows RALDH2 immunoreactivity in the atrium and at both right and left limbs of the sinus venosa. β-Gal activity is present in both atrium and sinus venosa, and additionally in the dorsomedial wall of the conotruncus. 10.5 dpc, RALDH2 immunoreactivity is present in the atria and the interventricular sulcus. β-Gal activity is present in the atria, interventricular sulcus, and the dorsomedial wall of the contruncus. 11.5 dpc, RALDH2 immunoreactivity has receded in the atria and is restricted to limited patches over the ventricular surface. β-Gal activity has receded in the atria and conotruncus and is completely absent from the ventricular surface. 12.5 dpc, RALDH2 immunoreactivity appears in both atria and ventricles. β-Gal activity is dramatically increased in the ventricles and in limited patches in the atria. 13.5 dpc, RALDH2 immunoreactivity persists in both atria and ventricles, although with less intensity than that observed at 12.5 dpc. Similarly, β-gal activity is less intense in the ventricles, but is completely absent from the atria.

pathway. Current studies are being carried out to distinguish between these possibilities.

Finally, the restriction of RA synthetic enzyme to the epicardium in the 12.5-dpc ventricle is not reflected in the response of the underlying myocardial tissue (Fig. 7). The simplest explanation for the discrepancy between epicardial RALDH2 expression and widespread ventricular activation of RA response is that other RA synthetic enzymes may be present in the heart by this stage. However, it is also possible that RA generated in the epicardium gains access to the trabeculae by circulation through newly formed coronary vessels. These form in the subepicardial space at 12.5 dpc (Viragh and Chalice, 1981).

A Role for RA in Myocardial Proliferation

The sudden appearance of RALDH2 in the ventricular epicardium at 12.5 dpc may reflect a role for RA in cell proliferation. Thickening of the ventricular wall is achieved through expansion of the compact zone, a layer of myocardial cells closely attached to the epicardium (Sucov *et al.*, 1994). In mice, this expansion is initiated at 12.5 dpc and coincides with a major surge in RA metabolism within the ventricular epicardium (Sucov *et al.*, 1994, and Fig. 7), suggesting that RA synthesis by the epicardium constitutes a physiological signal-inducing cell proliferation.

This role for RALDH2 in the epicardium is supported by studies in mice in which VCAM1, α4 integrin, and RXRα genes have been inactivated by targeted recombination. VCAM1 and α4 integrin embryos do not form a stable epicardial covering and die of heart failure sometimes compounded by a thin myocardium and septal defects (Kwee *et al.*, 1995; Yang *et al.*, 1995). RXRα knockout mice also die because of a hypoplastic myocardial wall and multiple ventricular septal defects (Sucov *et al.*, 1994; Kastner *et al.*, 1994, 1997b). Notably, compact zone cells in RXR$\alpha^{-/-}$ mice contain precocious sarcomeric organization, suggesting that defective RA signaling induces premature differentiation and growth arrest in the compact zone (Kastner *et al.*, 1994, 1997b), a phenotype also displayed by vitamin A-deficient rats. The present study reveals that proliferation of the compact zone induced by RA may be initiated as early as 12.5 dpc, concomitant with the onset of RALDH2 expression in the epicardium (Fig. 6).

A Role for RA Inactivation in Heart Development

Another means by which the activity of ligand may be delineated is through its inactivation. Here we have shown that in the developing 8.5-dpc heart, The RA-inactivating enzyme P450RA was present only in the endocardium (Fig. 4). The exclusion of RA from endocardium may be critical for proper formation of conotruncal cushions, since treatment with exogenous RA produces atrophy of conotruncal ridges and transposition of the great arteries (Nakajima *et al.*, 1996). P450RA is also expressed within rhombomere 2, in the first branchial arch, and in the foregut epithelium as well as in the tail region (Fig. 3; Fujii *et al.*, 1997). Severe tail truncations (Lohnes *et al.*, 1993), as well as head and heart defects, point to regions which are particularly sensitive to excess retinoids. Exogenous treatment with RA results in extension of the domains of P450RA expression (Fig. 5; Fujii *et al.*, 1997), suggesting a protective mechanism against inappropriate RA exposure.

Ligand Localization in Heart Development

This study establishes that RA signaling extends to virtually every aspect of the developing heart, with complex and shifting patterns of action that underscore the important role of localized RA presentation. Although RALDH2 is initially quite restricted, the developing heart is fully competent to respond to exogenously supplied ligand. This early competence may account for the dramatic detrimental effects of excess RA on cardiac development, in which programs of RA-responsive gene expression may be precociously activated.

The correspondence between RA-responsive structures and target sites for RA-related teratogenesis suggests that patterns of RALDH2 enzyme constitute physiological blueprints and instructions for sequential activation of RA-responsive gene expression during cardiac morphogenesis. A comprehensive description of RA signaling should pave the way for the dissection of the normal role of RA in individual events of cardiac development. Further elucidation of these processes awaits the development of spatially and temporally controlled perturbations of ligand synthesis and inactivation, through manipulation of the expression of the respective RALDH2 and P450RA genes.

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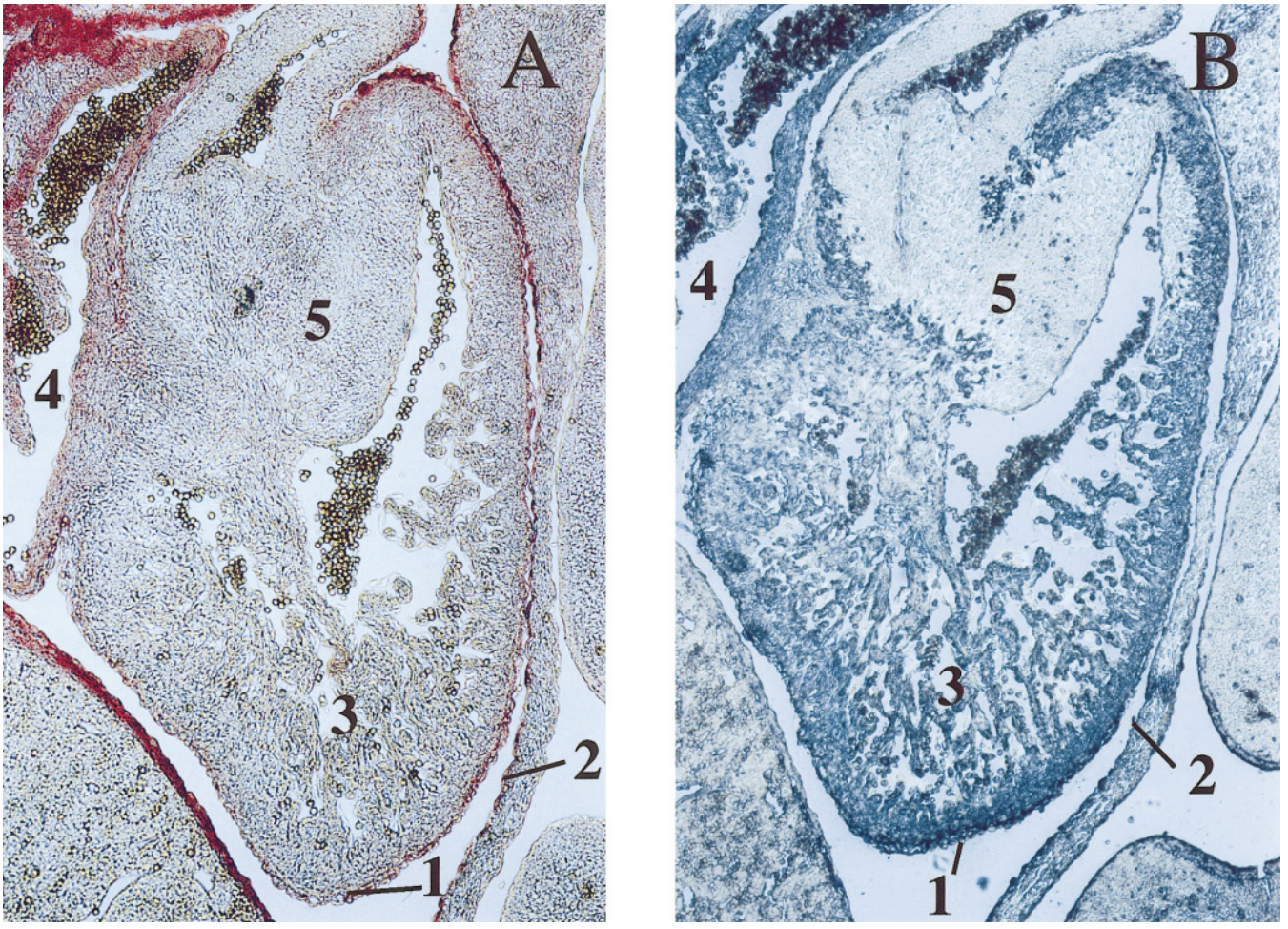

FIG. 7. Patterns of RALDH2 distribution and RA response in 12.5-dpc hearts. Paraformaldehyde-fixed embryos were embedded in paraffin and 5-μm sagittal sections were prepared. RALDH2 distribution and RA response were investigated with antibodies raised against RALDH2 and β-galactosidase, respectively. Positive tissues were detected by the sequential addition of IgG–biotin and then avidin–alkaline phosphatase-conjugated antibodies. (A) RALDH2 expression is observed in epicardial (1) and pericardial (2) layers, as well as in limited areas of the atrium (4) using Fast Red as a substrate for alkaline phosphatase. Note that neither trabeculated ventricular myocardium (3) nor cushion tissue (5) expresses RALDH2. (B) RA response, as indicated by β-gal activity, is observed in epicardial (1) and pericardial (2) layers, as well as in the atrium (4), using BCIP/NBT as substrate for alkaline phosphatase. In contrast to RALDH2, β-gal activity is found in the trabeculated myocardium, but is absent from most of cushion tissues (5).

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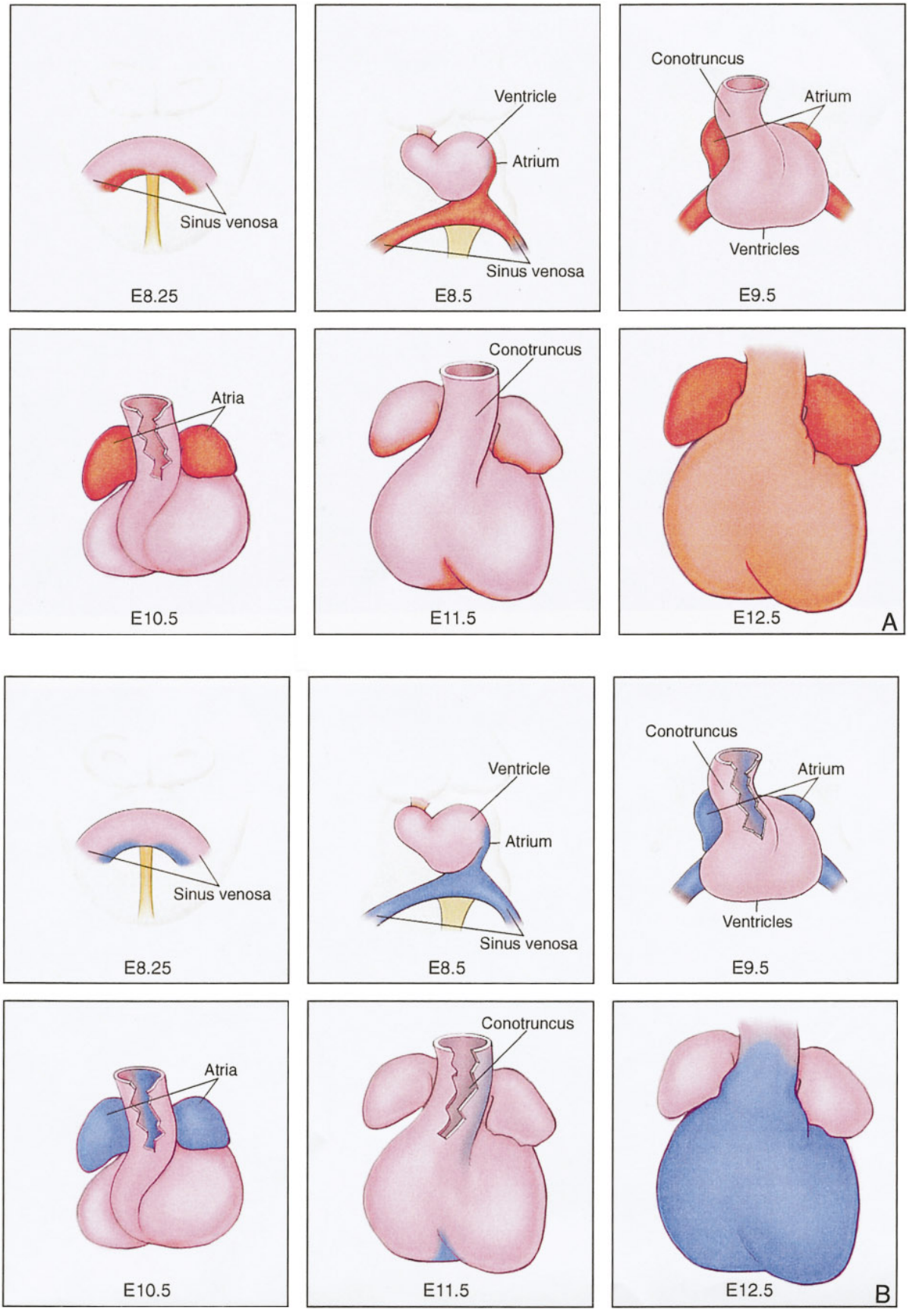
FIG. 8. Summary of RALDH2 distribution and RA responsiveness in the developing mouse heart. A schematic representation of RALDH2 enzyme distribution (A) and RARE-lacZ transgene expression (B) at different stages of mouse heart development.


Sinus venosa
E8.25
Ventricle
Atrium
Sinus venosa
E8.5
Conotruncus
Atrium
Ventricles
E9.5
Atria
E10.5
Conotruncus
E11.5
E12.5
A
Sinus venosa
E8.25
Ventricle
Atrium
Sinus venosa
E8.5
Conotruncus
Atrium
Ventricles
E9.5
Atria
E10.5
Conotruncus
E11.5
E12.5
B

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