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Sequential Programs of Retinoic Acid Synthesis in the Myocardial and Epicardial Layers of the Developing Avian Heart

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Endogenous patterns of retinoic acid (RA) signaling in avian cardiac morphogenesis were characterized by localized expression of a key RA-synthetic enzyme, RALDH2, which displayed a biphasic pattern during heart development. RALDH2 immunoreactivity was initially apparent posterior to Hensen's node of stage 5–6 embryos and subsequently in somites and unsegmented paraxial and lateral plate mesoderm overlapping atrial precursors in the cardiogenic plate of stage 9– embryos. Initial RALDH2 synthesis in the posterior myocardium coincided with activation of the *AMHC1* gene, a RA-responsive marker of inflow heart segments. A wave of RALDH2 synthesis then swept the myocardium in a posterior-to-anterior direction, reaching the outflow tract by stage 13, then fading from the myocardial layer. The second phase of RALDH2 expression, initiated at stage 18 in the proepicardial organ, persisted in migratory epicardial cells that completely enveloped the heart by stage 24. Early restriction of RALDH2 expression to the posterior cardiogenic plate, overlapping RA-inducible gene activation, provides evidence for commitment of posterior avian heart segments by localized production of RA, whereas subsequent RALDH2 expression exclusively in the migratory epicardium suggests a role for the morphogen in ventricular expansion and morphogenesis of underlying myocardial tissues.

Key Words: **atria; ventricles; retinoic acid; RALDH2; epicardium.**

INTRODUCTION

In the developing vertebrate embryo, retinoic acid (RA) is synthesized by a series of oxidative reactions that convert the dietary precursor vitamin A to active retinoids (Means and Gudas, 1995). A predominant view holds that these compounds act as morphogens by diffusing to surrounding cells to instruct the fate of different target tissues. According to this view the diverse morphogenic actions of RA are mediated by concentration gradients and by combinations of different members of a family of RA-binding nuclear receptor proteins, RARs and RXRs, that form heterodimers and target DNA regulatory sequences to modify gene expression (Mangelsdorf *et al.*, 1995). However, despite considerable effort, it has been technically difficult to document gradients of RA as well as to derive specific roles for the various retinoid receptors based on their abundance and distribution (Dickman *et al.*, 1997). The pleiotropic effects of retinoids likely cannot be explained exclusively on the basis of dose profiles generated by diffusion from centralized sources or by selective expression of different receptor proteins.

The availability of mice harboring RA indicator transgenes as surrogates for direct RA detection (Rossant *et al.*, 1991) and the cloning of a key RA-synthetic enzyme RALDH2 (Zhao *et al.*, 1996) have made possible a direct comparison between the territories of RA action and RA synthesis. As demonstrated by Moss *et al.* (1998), responsiveness to RA maintains a remarkable correspondence

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with domains of RALDH2 expression, suggesting that the morphogenetic action of RA during development is, as a general rule, more often regulated by localized ligand production rather than by diffusion from critical centers or hot spots such as in the eye or in the spinal cord (Colbert *et al.*, 1993; McCaffery *et al.*, 1995). The focus of retinoid research has thus been expanded from concentration gradients and distribution of nuclear receptors to regulation of localized RA synthesis (Moss *et al.*, 1998; Xavier-Neto *et al.*, 1999).

We recently described distinct and dynamic morphogenetic fields of RA signaling in the developing mouse heart, in sinoatrial structures which first activate RA-induced transcription at 8.25 dpc, followed by the dorsomedial wall of the conotruncus at 8.5 dpc and by the ventricles at day 12.5 dpc (Moss *et al.*, 1998; Xavier-Neto *et al.*, 1999). In the present study we expanded this characterization to the avian system, which allowed us to evaluate the evolutionary significance of the RA morphogenetic fields described in the mouse and to extend our analysis to later stages of cardiac development. Using an antibody against the RA-synthetic enzyme RALDH2, we mapped territories of RA synthesis in the avian heart, exploiting the RA-inducible *AMHC1* gene as an early marker of atrial development (Yutzey *et al.*, 1994). Profiles of RA synthesis indicate that avians and mice employ similar strategies for RA signaling in the developing heart. Later restriction of RALDH2 expression to the migratory epicardium identifies RA as a candidate signaling molecule in epicardial induction of ventricular myocardial proliferation and development of the coronary circulation and provides a novel molecular model for signaling in these critical tissue interactions.

MATERIALS AND METHODS

Chicken and Quail Embryos

Fertilized unincubated chicken and quail eggs were obtained from Spafas and Truslow Farms, respectively. The eggs were incubated at 37.5°C and 98% relative humidity and embryos were harvested at indicated stages.

In Situ Hybridization and Immunohistochemistry

Whole-mount *in situ* hybridization of embryos and dissected hearts was performed according to established protocols (Wilkinson, 1992) using AMHC1 antisense probes as previously described (Yutzey *et al.*, 1994). For immunohistochemical analysis, rabbit polyclonal antibodies raised against RALDH2 were used at dilutions of 1:400 to 1:200. The specificity of the RALDH2 antibody in the avian system has previously been demonstrated elsewhere (Berggren *et al.*, 1999). Whole-mount samples were processed as previously described (Dent *et al.*, 1989) with horseradish peroxidase-labeled donkey anti-rabbit IgG (Amersham NA 934; 1:1000) as secondary antibody. Stains were performed with DAB as a substrate with or without nickel, which gave chicken and quail positive tissues a brown or black color, respectively. Paraffin sections were generated by standard methods (Sassoon and Rosenthal, 1993) and immunostained with the RALDH2 antibody and an anti-rabbit IgG alkaline phosphatase conjugate using fast red (Sigma) as a substrate (Neville *et al.*, manuscript in preparation). For double staining, embryos were first submitted to *in situ* hybridization and subsequently fixed in Dent's fixative, then immunostained with RALDH2 antibody as described above. Control samples omitting the primary antibody were negative.

Image Analyses and Processing

Embryos were photographed in whole mount on a Nikon dissecting microscope (SMZ-2T) using 64 ASA tungsten slide film. Stained paraffin sections were photographed on a Zeiss Axiophot microscope using 100 ASA film. Images were digitized with a Polaroid Sprint Scan 35 slide scanner.

RESULTS

Early RALDH2 Expression in Avian Heart Development

We analyzed the time course of RALDH2 expression in the quail embryo, from Hamilton and Hamburger (1951) stages 8 through 24, using an anti-RALDH2 antibody as previously described (Moss *et al.*, 1998). At stage 8, RALDH2 enzyme levels were high in the unsegmented paraxial mesoderm and in the first three somites (Fig. 1A, see also below). The lateral mesoderm was also positive in a region broadly encompassing the middle third of the embryo. At this stage the anterior limit of RALDH2 immunoreactivity in the lateral mesoderm had already expanded slightly above the first somite. At stage 9 both paraxial and lateral mesoderm displayed more organized features with the formation of seven somites and with fusion of ventricular precursors at the midline. At this stage most somites, unsegmented paraxial mesoderm, and lateral plate mesoderm continued to display high levels of RALDH2 immunoreactivity, while the enzyme levels faded in the anteriormost somite (Fig. 1B). At stage 10 the newly formed heart displayed restriction of RALDH2 expression to the posterior, unfused myocardium, coinciding with the distribution of atrial precursors (Rosenquist and deHaan, 1966). In contrast, the fused myocardium representing ventricular and outflow precursors did not contain RALDH2 (Fig. 1C). At subsequent stages RALDH2 immunoreactivity was restricted to increasingly shorter lengths of unsegmented paraxial mesoderm, while maintaining strong expression in the somites. At stage 11 the heart established a rightward loop and the restriction of RALDH2 expression to sinoatrial precursors became more apparent (Fig. 1D).

In more developed embryos RALDH2 immunoreactivity progressed anteriorly, reaching the common ventricle by stage 12+ (Fig. 2A) and the first third of the conotruncus by stage 13 (Fig. 2B) and eventually including the whole heart by stage 13 (Fig. 2C) or stage 14 (data not shown). This graded wave of RALDH2 expression was restricted to the myocardium, as shown by RALDH2 staining of coronal sections through a stage 18 quail embryo (Fig. 2D).

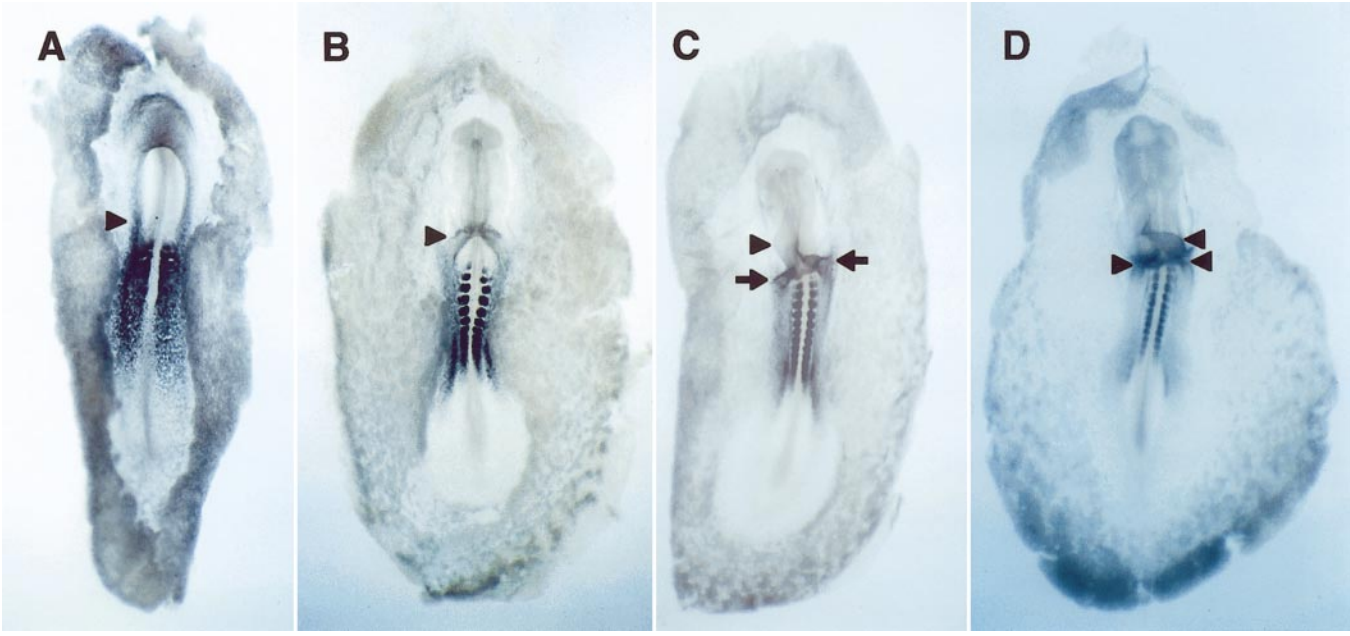


FIG. 1. RALDH2 immunoreactivity in the early quail embryo. (A) Stage 8–Hamburger and Hamilton (HH). Note that RALDH2 immunoreactivity in the lateral mesoderm extends anterior to the anterior border of the first somite (arrowhead). (B) Stage 9 HH. Note that RALDH2 immunoreactivity in the heart is restricted to posterior heart precursors (arrowhead). (C) Stage 10 HH. RALDH2 expression persists in the posterior, unfused myocardium (arrows). The fused myocardium of the outflow tract (arrowhead) does not express RALDH2. (D) Stage 11 HH. RALDH2 expression in the heart is restricted to sinoatrial precursors (arrowheads).

RALDH2 Enzyme Overlaps RA-Inducible AMHC1 Gene Expression in the Avian Myocardium

To further investigate the relationship between RALDH2 expression and axial patterning in the developing avian myocardium, we exploited the chick *AMHC1* gene as a marker of posterior specification of cells destined to form inflow heart segments (Yutzey *et al.*, 1994) and performed a developmental comparison of RALDH2 immunoreactivity and *AMHC1* gene expression at early stages of chick heart development. At stage 6, RALDH2 was present in posterior embryonic regions, creating a sharp anteroposterior boundary at the level of Hensen's node (Fig. 3A). At stage 8, RALDH2 immunoreactivity was intense in the lateral plate mesoderm as well as in the unsegmented paraxial mesoderm and two posterior somites, but down regulated in the first two anterior somites (Fig. 3B), a pattern also seen in the quail (see Figs. 1B and 1C). Anterior expansion of RALDH2 immunoreactivity in the lateral mesoderm reached the most posterior segment of the cardiogenic plate by stage 9 (Fig. 3C), extending into the prospective left ventricle by stage 12 (Fig. 3D).

Posterior heart precursors could first be distinguished from their anterior counterparts by expression of the *AMHC1* gene around stages 8 and 9, as revealed by *in situ* mRNA hybridization (Fig. 4A). *AMHC1* transcripts in more mature embryos reflected the morphogenesis of the posterior segment of the heart, with movement of left and right segments toward the midline (Fig. 4B), and their subsequent fusion to generate the atrial chamber at stage 11 as described by Yutzey *et al.* (1994). Notably, later *AMHC1* gene expression expanded anteriorly to include the atrioventricular canal and the prospective left ventricle by stage 12 (Fig. 4C), coinciding with the cardiac distribution of RALDH2 immunoreactivity at the same stage (Fig. 3D).

To delineate more precisely the region of RALDH2 expression that coincided with the earliest onset of *AMHC1* activation, we performed *in situ* hybridization analysis of a stage 9 embryo with an *AMHC1* antisense probe, followed by immunostaining of the same embryo with a RALDH2 antibody. Figure 5 shows *AMHC1* expression overlapped with that of RALDH2 exclusively in a segment of the cardiogenic plate that is fated to give rise to sinus venosa and atria (Rosenquist and de Haan, 1966). Thus in early stages of avian cardiogenesis, *AMHC1* expression represents a *bona fide* marker of presumptive atrium, since it was strictly segregated to posterior cardiac precursors and was never observed anteriorly, even after prolonged exposure (data not shown).

RALDH2 Expression Marks Migratory Epicardial Precursors

As RALDH2 immunoreactivity slowly faded from the myocardial layer, a new focus of cardiac RALDH2 expression was initiated. In stage 18 quail embryos, the grape-like

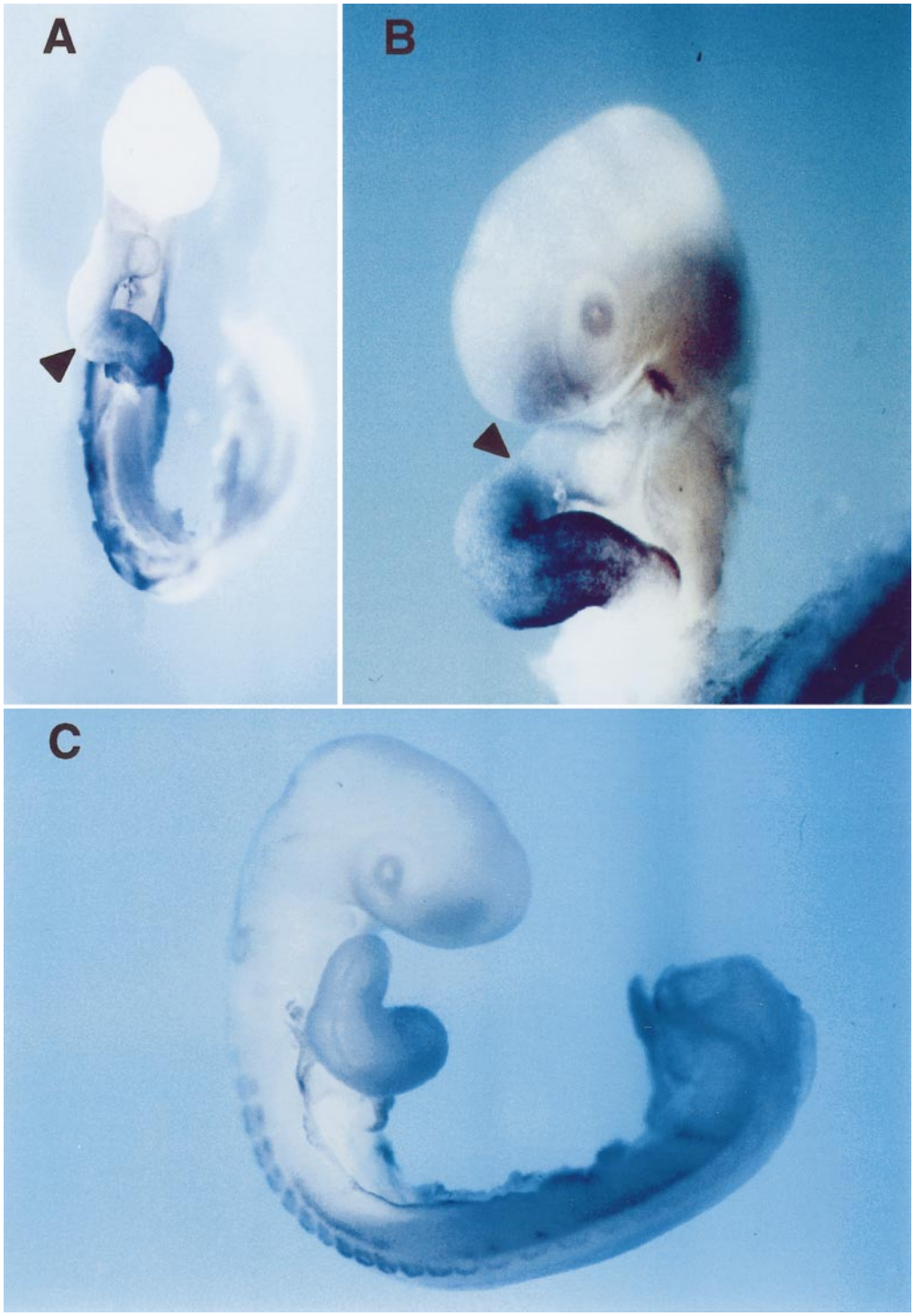


FIG. 2. RALDH2 immunoreactivity during later stages of quail cardiogenesis. (A) Stage 12+ HH. RALDH2 immunoreactivity in the quail embryo reaches the common ventricle (arrowhead). (B) Stage 13 HH. RALDH2 in the lower conotruncus. (C) Stage 13 HH. RALDH2 in the entire myocardium. (D) RALDH2 in a coronal section of a stage 18 quail. Note staining of myocardium in the common atrium and in the left wall of the common ventricle.

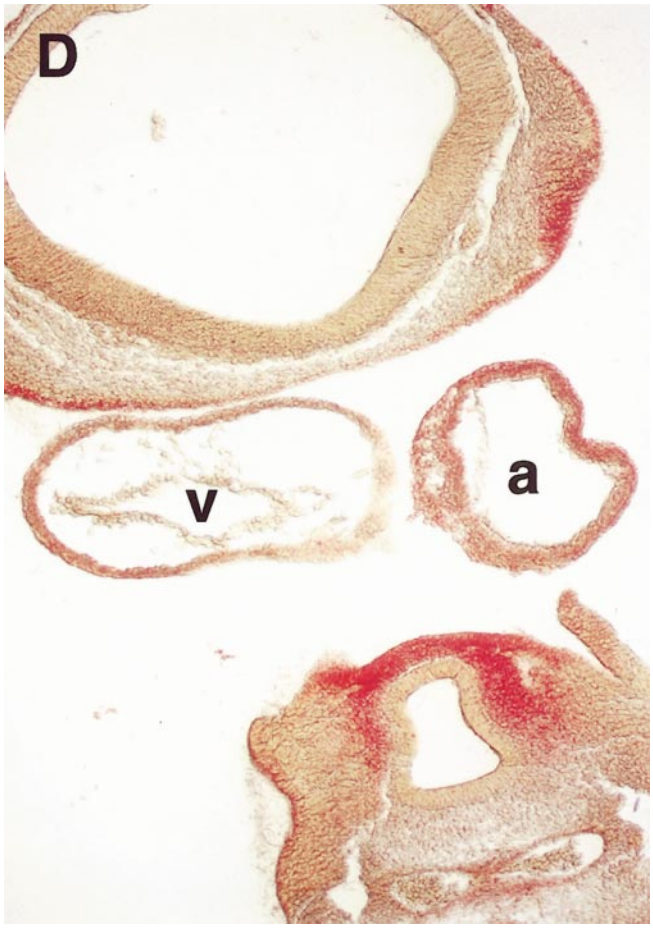


FIG. 2—*Continued*

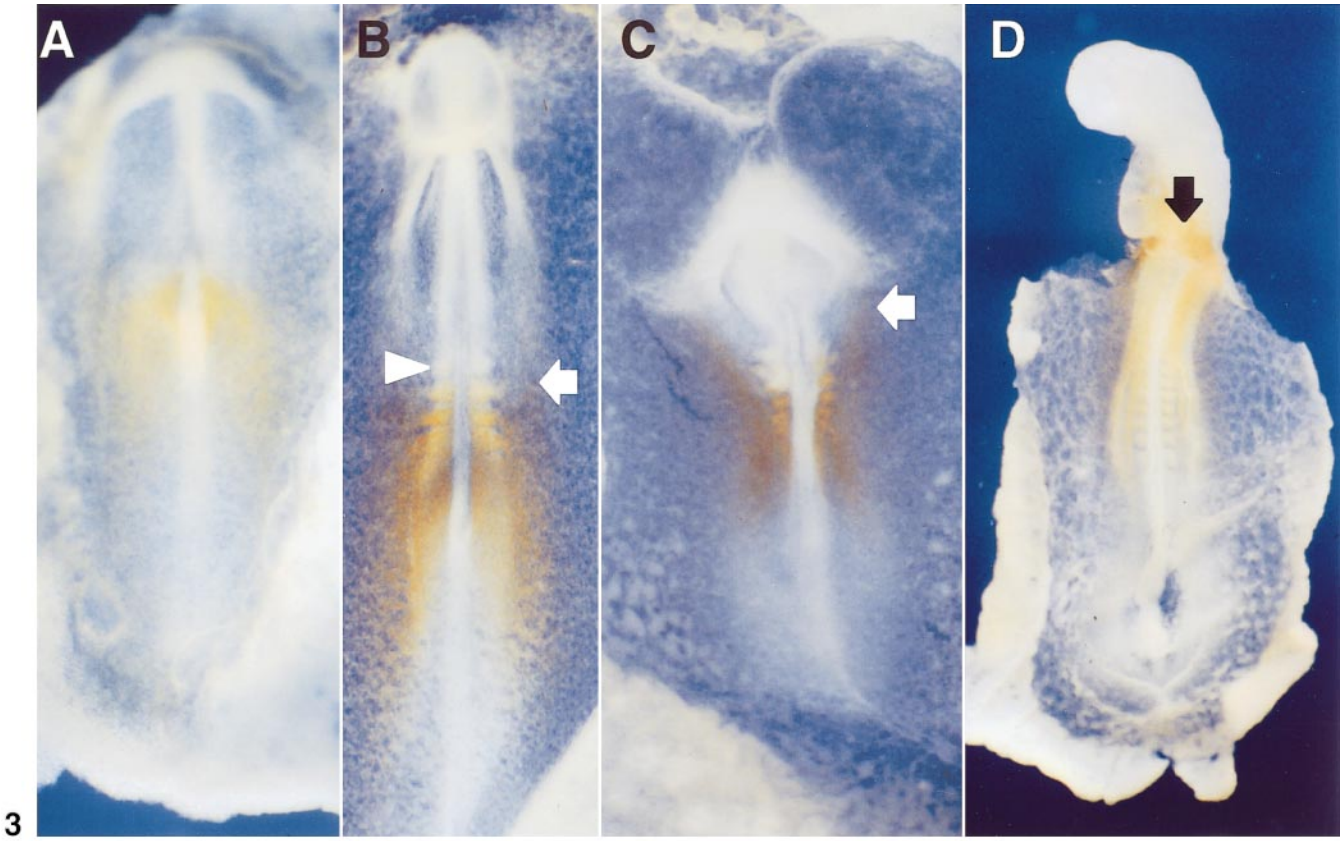
cells of the epicardial organ, located in the septum transversum between the heart and the liver bud (Poelmann *et al.*, 1993), stained intensely with RALDH2 antibody (Fig. 6A, arrow). Simultaneously, another center of RALDH2 immunoreactivity appeared at the atrial surface (Fig. 6A, arrowhead). RALDH2-positive cells quickly enveloped the common atrium, while positive cells in the migratory epicardium spread over the ventricular surface of the stage 19 heart (Fig. 6B), progressively covering the prospective left (Fig. 6C) and right (Fig. 6D) ventricles by stages 20 and 21, respectively. At stage 22, most of the surface of the heart was already enveloped by the spreading RALDH2-expressing epicardium, with only a limited area of myocardium left uncovered in the ventral face of the truncus (Fig. 6E), revealing the absence of RALDH2 from the myocardial layer at this stage. Complete envelopment of the heart by epicardial cells was attained by stage 24, as seen in isolated hearts and in whole embryos with dissected pericardia (Figs. 6F and 6G), while a negative control heart did not show significant staining (Fig. 6H). A parasaggital section through a stage 24 quail embryo confirmed the epicardial restriction of RALDH2. At this stage the entire myocardium was devoid of RALDH2 (Fig. 6I).

The ascending wave of RALDH2 expression in the epicardium was invariably initiated after its expression in the myocardium was established. The spatial–temporal relationship between myocardial and epicardial expression of the RALDH2 enzyme was variable, however. Some isolated hearts (see Figs. 6B, 6C, 6D, 6E, and 6I) and sections (not shown) displayed segments with exclusively myocardial or epicardial expression, while in others epicardial and myocardial staining overlapped, predominately in presumptive left and right ventricles (Figs. 6C and 6D and data not shown). In contrast, the upper truncus was usually devoid of myocardial enzyme immunoreactivity before envelopment by the RALDH2-expressing epicardium (see Fig. 6C).

DISCUSSION

RALDH2 Synthetic Programs Reveal Novel Insights into RA Action in Cardiac Development

A role for RA in the morphogenesis of atrial chambers in the developing vertebrate heart has been suggested by the teratogenic effects of RA excess and deficiency (Heine *et al.*, 1985; Osmond *et al.*, 1991; Stainier and Fischman, 1992; Dersh and Zile, 1993; Yutzey *et al.*, 1994, 1995; Twal *et al.*, 1995; Kostetskii *et al.*, 1999). These studies have traditionally utilized exogenous retinoid administration (Osmond *et al.*, 1991; Stainier and Fischman, 1992; Yutzey *et al.*, 1994) or RA-deficient states generated by vitamin A deprivation (Heine *et al.*, 1985; Dersh and Zille, 1995) or by inhibition of RA biosynthesis (Costaridis *et al.*, 1996; Stratford *et al.*, 1996). More recently, expression of constitutively active and dominant-negative forms of RA receptors has also been incorporated into the arsenal of techniques in RA biology (Damm *et al.*, 1993). The relevance of these strategies, however, depends on whether the tissues affected are actively involved in RA signaling at the time of the intervention. Studies utilizing exogenous RA or modified RA receptor forms are particularly vulnerable to artifact, since it is clear that RA signaling can trigger nonphysiological developmental programs when activated ectopically. On the other hand, experimental pertubations involving vitamin A deprivation are restricted by a lack of temporal and spatial control, revealing only the consequences of chronic retinoid deprivation (Smith and Dickman, 1997). Clearly, studies on the effects of RA are most clearly interpretable by monitoring endogenous profiles of retinoid action at the various stages of development. Early attempts to do so utilized HPLC to measure the concentrations of retinoids in dissected tissues (Napoli, 1986; Horton and Maden, 1995). However, HPLC methods are not sensitive enough to give adequate spatial resolution, and as a result, HPLC analyses produced reliable data only in more developed embryos, after most critical events in organogenesis had already taken place. To circumvent these difficulties Maden *et al.* (1998) associated HPLC analyses with bioassays, in which



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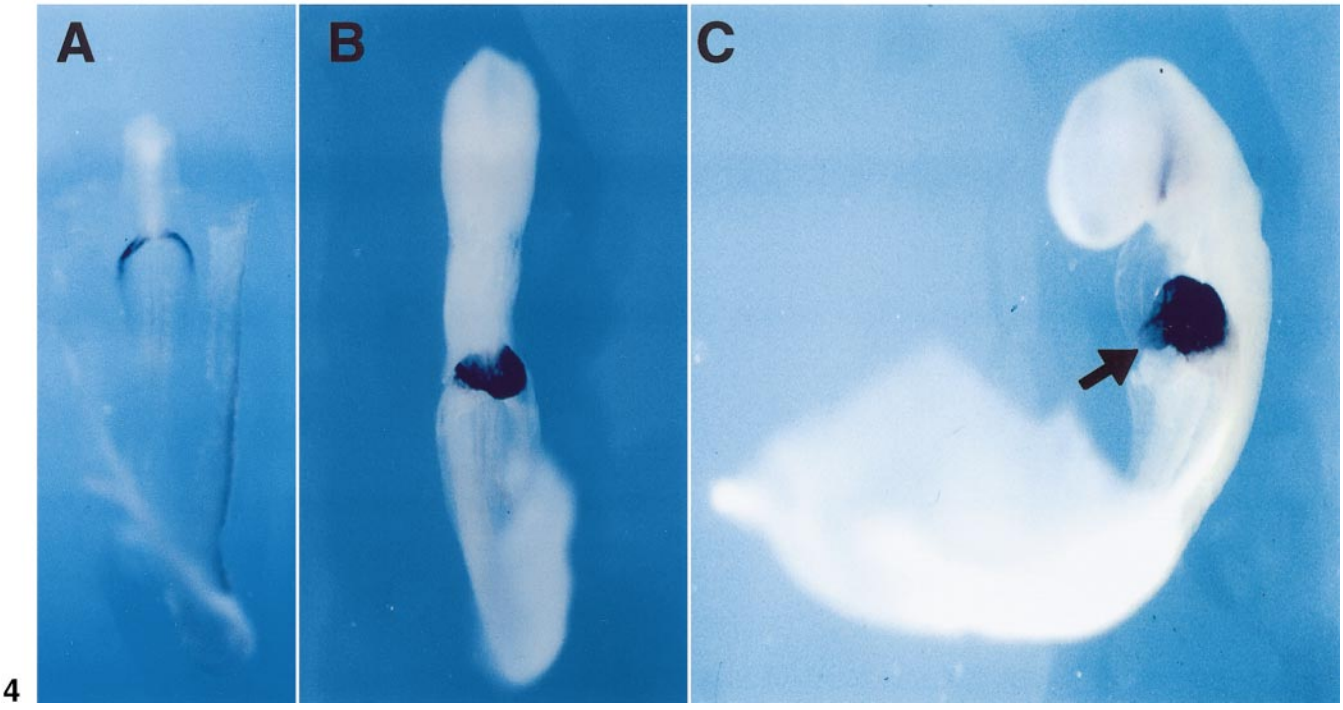

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FIG. 3. Early RALDH2 expression in chick cardiac inflow segments. (A) RALDH2 stain of a stage 6 HH chick embryo. (B) Stage 8 HH embryo. Note the beginning of anterior expansion of RALDH2 immunoreactivity in the lateral mesoderm (arrow) and the down regulation of RALDH2 expression in the first two somites (arrowhead). (C) Stage 9– HH embryo. Note marked anterior extension of RALDH2

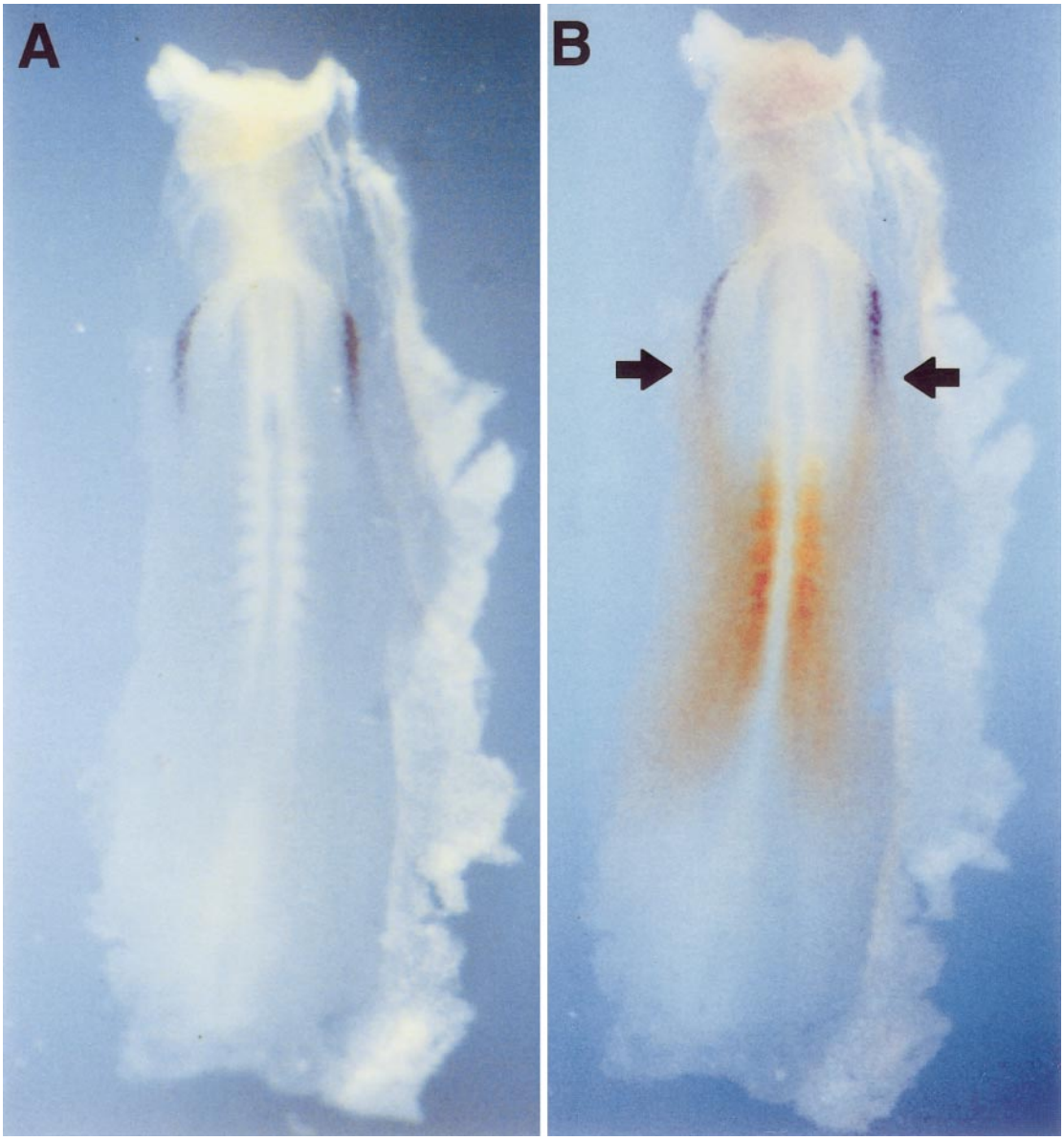

FIG. 5. *AMHC1* activation in RALDH2 synthetic tissue. (A) *AMHC1 in situ* hybridization of a stage 9– HH embryo showing the location of posterior heart precursors in the cardiogenic plate. (B) RALDH2 stain of the same embryo. Note how anterior extension of RALDH2 immunoreactivity in the lateral mesoderm reaches posterior cardiac precursors expressing the *AMHC1* gene (arrows).

immunoreactivity overlapping posterior segments of the cardiogenic plate (arrow). (D) RALDH2 immunoreactivity in a stage 12 HH embryo. Note extension of RALDH2 immunoreactivity to the prospective left ventricle (arrow).

FIG. 4. Onset of chick *AMHC1* expression visualized by *in situ* hybridization. (A) *AMHC1* expression in a stage 11 HH chick embryo showing movement of posterior cardiac primordia toward the midline. (B) *AMHC1* expression in a stage 11+ HH embryo showing fusion of paired posterior heart precursors into a midline sinoatrial compartment. (C) *AMHC1* expression in a stage 12 HH embryo showing extension of *AMHC1* expression to the prospective left ventricle (arrow).

microdissected tissue is placed in contact with a sensitive RA reporter cell line, to generate maps of RA levels at various stages of chicken development. The RA levels in those studies correlate well with the distribution and intensity of RALDH2 immunoreactivity as reported here.

Analysis of RALDH2 immunoreactivity provides more detailed information about RA synthesis than has been previously possible. For instance, RALDH2 immunostaining has revealed the dynamic nature of RA signaling during heart development, represented in myocardial and epicardial waves of RALDH2 expression, and the existence of a transient rostrocaudal gradient of RALDH2 developing in stage 9 somites. Interestingly, RALDH2 distribution can also explain the discrepancy between RA content as measured by HPLC and inferred by a reporter cell bioassay in stage 24 hearts, when the heart produced more positive cells than expected from its measured content of RA (Maden *et al.*, 1998). At stage 24 only the outer cardiac cell layer, the epicardium, expresses RALDH2 (see Fig. 2), which accounts for the low cardiac levels of RA when normalized by tissue weight. In the bioassay, however, epicardial cells are in direct contact with reporter cells, generating a microenvironment rich in RA and an efficient activation of the RA-sensitive transgene despite the fact that total levels of RA in the whole heart are rather low by stage 24. Thus, immunolocalization of RALDH2 has proven a valuable method to characterize endogenous profiles of RA signaling during embryogenesis. It is likely that in future studies, RALDH2 immunolocalization will be used in conjunction with other techniques to provide a better assessment of the role displayed by RA in heart development.

Evolutionary Conservation of RALDH2 Expression Patterns in Heart Development

The detailed comparison presented here indicates that the avian heart shares the same basic developmental profiles of RA action observed in early mammalian cardiogenesis, with an initial localization to cardiac inflow and a subsequent progression into ventricles and outflow tract. Although the patterns of RALDH2 expression in the avian heart are similar to those of mice, there are important differences nonetheless. In mice, the myocardial expression of RALDH2 is limited to sinus venosa and atrium, while the ventricular myocardium is only indirectly exposed to RA late in development when migrating epicardial cells expressing RALDH2 envelop the ventricles from a posterior position in the septum transversum (Moss *et al.*, 1998). In avians, by contrast, the myocardial wave of RALDH2 immunoreactivity sweeps the heart in a posterior to anterior direction to include atria, prospective ventricles, and outflow tract (Fig. 1).

The functional significance of this early exposure to RA signaling in avian ventricular tissue remains to be established. Extension of *AMHC1* expression in the ventricle was apparent in Northern blots from embryonic day 7 chicken embryos, although not detectable by embryonic day 11 with this method (Yutzey *et al.*, 1994). However *AMHC1* transcripts have also been observed in whole-mount *in situ* mRNA hybridizations following prolonged incubation with chromogenic substrates (David Bader, personal communication). The late expansion of *AMHC1* expression into the prospective left ventricle correlates well with progression of RALDH2 immunoreactivity into the prospective left ventricles of stage 12 embryos, suggesting that exposure to RA signaling at late stages could activate the *AMHC1* gene in some ventricular cells. Nevertheless, it is clear that these ventricular cells never express a full-blown atrial phenotype, since commitment to atrial and ventricular phenotypes has already taken place by stage 8 (Yutzey *et al.*, 1995). Early commitment to more anterior cell fates could explain the lack of conversion to atrial types in response to the anterior expansion of RALDH2 expression either in the myocardium or in the epicardium. This hypothesis is also consistent with the narrow window of opportunity available for posterior transformation of the heart by RA in mice (Xavier-Neto *et al.*, 1999).

RALDH2 is also expressed in the epicardial cells of both avian and mammalian hearts. In quail, RALDH2 immunoreactivity in whole-mount embryos describes in three dimensions the enveloping of the heart between stages 18 and 24, precisely matching the time frames established in previous studies (Poelmann *et al.*, 1993; Peeters *et al.*, 1995). The continuous growth of the epicardial sheet in avian ventricles contrasts with the patchy and irregular spreading of epicardial cells in the mouse (Moss *et al.*, 1998). Notably, both quail and chicken embryos display some degree of overlap between the territories of myocardial and epicardial expression of RALDH2. One possibility is that myocardial fibroblast precursors delaminating from the epicardium (Gittenberger-de Groot *et al.*, 1998) retain RALDH2 expression during their epithelial–mesenchymal transformation and contribute to RA synthesis in the ventricles. Although the precise role of RA in ventricular development remains to be defined, the similarity of RALDH2 expression patterns in both chicken and quail, as well as in the lizard *Hemiergis* (M.D.S., unpublished observations), indicates that synthesis of RA in the growing epicardium is an evolutionarily conserved feature of heart development in amniotes.

Early RALDH2 Expression Marks the Partition of the Heart into Atrial and Ventricular Compartments

The avian system in this study has allowed us to document the progression of RALDH2 expression in more detail than previously reported in the mouse (Niederrheiter *et al.*, 1997; Moss *et al.*, 1998; Xavier-Neto *et al.*, 1999). It has also permitted the exploitation of an endogenous RA-inducible atrial marker, the *AMHC1* gene, rather than a transgene, to describe the relationship between RALDH2 expression, RA responsiveness, and development of cardiac inflow struc-

tures. The dynamic changes in RALDH2 immunoreactivity described here suggest a molecular mechanism whereby RA signaling controls specification and commitment to the sinoatrial phenotype. In agreement with previous studies in the mouse (Niederreiter *et al.*, 1997; Xavier-Neto *et al.*, 1999), avian cardiac precursors are likely to receive anteroposterior information by RA signaling for the first time at mid- to late gastrulation (stages 5–6), when RALDH2 is first expressed in the mesoderm posterior to Hensen's node (Fig. 3A) and anterior cardiac precursors have already migrated anterolaterally (Tam *et al.*, 1997). Therefore, only posterior cardiac precursors, the cells that are fated to give rise to the inflow segment of the heart (Rosenquist and DeHaan, 1966), are likely to have migrated through an active field of RA signaling or to be in range of RA diffusion. Thus, either cell-autonomous synthesis of RALDH2 or exposure to nearby RA at stages 5 to 6 would trigger the inflow phenotype in posterior cardiac precursors. This stage interval is consistent with the temporal window when undifferentiated cardiogenic mesoderm is specified to atrial or ventricular fates (Yutzey *et al.*, 1995).

As shown in Fig. 3, RALDH2 immunoreactivity in the lateral mesoderm progresses from an initial anteroposterior border at the level of Hensen's node at stage 6 to reach the posterior segment of the cardiogenic plate by stage 8. This RALDH2-expressing domain coincides with the posterior segment of the cardiogenic plate that is fated to give rise to inflow structures of the heart (Rosenquist and deHaan, 1966), marked in the avian embryo by overlapping *AMHC1* expression, suggesting that local production of RA in posterior heart precursors initiates expression of the *AMHC1* gene and irreversibly commits them to the atrial fate (Yutzey *et al.*, 1995). The correlation between expression of RALDH2, an RA synthethic enzyme, and *AMHC1*, a RA-inducible gene, is compatible with the observed induction of the *AMHC1* gene in anterior heart precursors following systemic administration of RA to chicken embryos (Yutzey *et al.*, 1994).

These data give rise to a model in which the interplay between cardiac-specific transcription factors and RA signaling determines a posterior cell fate, while anterior fates (ventricles and conotruncus) are specified in the absence of RA. This model is consistent with both the timing of commitment to anteroposterior cell fates in the heart and the cardiac outcomes of RA excess and insufficiency observed in quail, chicken, and zebrafish (Heine *et al.*, 1985; Twal *et al.*, 1995; Osmond *et al.*, 1990; Stainier and Fishman, 1992). In mice, RALDH2 and the endogenous response to RA colocalize in the sinus venosa and atria of the developing mouse heart, suggesting that localized production of RA is key to the development of cardiac inflow structures (Moss *et al.*, 1998). More recently, we have shown that a human alkaline phosphatase (HAP) reporter transgene driven by the quail slow myosin heavy chain (SMyHC3) promoter specifically labeled the sinoatrial population from the earliest stages of murine cardiogenesis up to adulthood (Xavier-Neto *et al.*, 1999). Exposure to exogenous RA led to atrialization of the heart with corresponding increases in SMyHC3–HAP activity, whereas inhibition of RA synthesis with disulfiram ablated the atrial chamber, reduced SMyHC3–HAP activity, and resulted in embryonic lethality (Xavier-Neto *et al.*, 1999). Other studies in which RA signaling was suppressed either by administration of a RA panantagonist to cultured embryos (Chazaud *et al.*, 1999) or by the targeted ablation of the *RALDH2* gene (Niederreither *et al.*, 1999) are consistent with our observations, since in both cases the embryos are missing the atrial chamber and display enlarged ventricles. RA is also required in the mouse embryo for the determination of left–right asymmetry (Chazaud *et al.*, 1999); however, loss of the unilateral regression of the atrium and cardinal vein (sinus venosus region) in the presence of RA antagonist may be secondary to the perturbation of the early wave of RA expression in atrial precursors. Collectively, these studies indicate that early specification of the sinoatrial compartment in vertebrates is controlled by localized synthesis of RALDH2. This conclusion is in agreement with an early role for RA in posterior patterning in the developing myocardium (Moss *et al.*, 1998; Xavier-Neto *et al.*, 1999; Niederreither *et al.*, 1997) and could explain its teratogenic effects on ventricles and outflow tract when ectopically administered at critical embryonic stages (Xavier-Neto *et al.*, 1999).

RALDH2 Expression Patterns Reveal RA Signaling in the Epicardium

Previous studies have uncovered the critical importance of the epicardial layer in heart development. Chick–quail chimeric experiments involving transplantation of the proepicardial organ have demonstrated that migratory epicardial cells contribute a novel population to the myocardial wall and to the atrioventricular cushions (Grittenberger-de Groot *et al.*, 1997), in agreement with the results from studies using adenoviral labeling of proepicardial cells (Dettman *et al.*, 1998). Smooth muscle cells and fibroblasts of the coronary arteries also derive from the epithelial–mesenchymal transformation of epicardial-derived cells (Dettman *et al.*, 1998; Vranken Peeters *et al.*, 1999; Reese *et al.*, 1999). The cardiopathic effects of targeted disruptions in genes encoding adhesion proteins VCAM-1 and α4 integrin as well as specific RA receptors support critical roles for epicardial RA signaling in ventricular myocardial proliferation and development of the coronary circulation. Inactivation of the former two genes resulted in embryonic lethality compounded by lack of stable epicardial covering, lack of coronary vessels, thinning of the ventricular wall, and ventricular septal defects (Kwee *et al.*, 1995; Yang *et al.*, 1995). RXRα-null animals also displayed a hypoplastic ventricular wall associated with precocious differentiation of compact zone cells and extensive ventricular septal defects (Sucov *et al.*, 1994; Kastner *et al.*, 1997), a phenotype also characteristic of vitamin A-deprived rats (Wilson and Warkani, 1949; Kaster *et al.*, 1997). The epicardial origin of

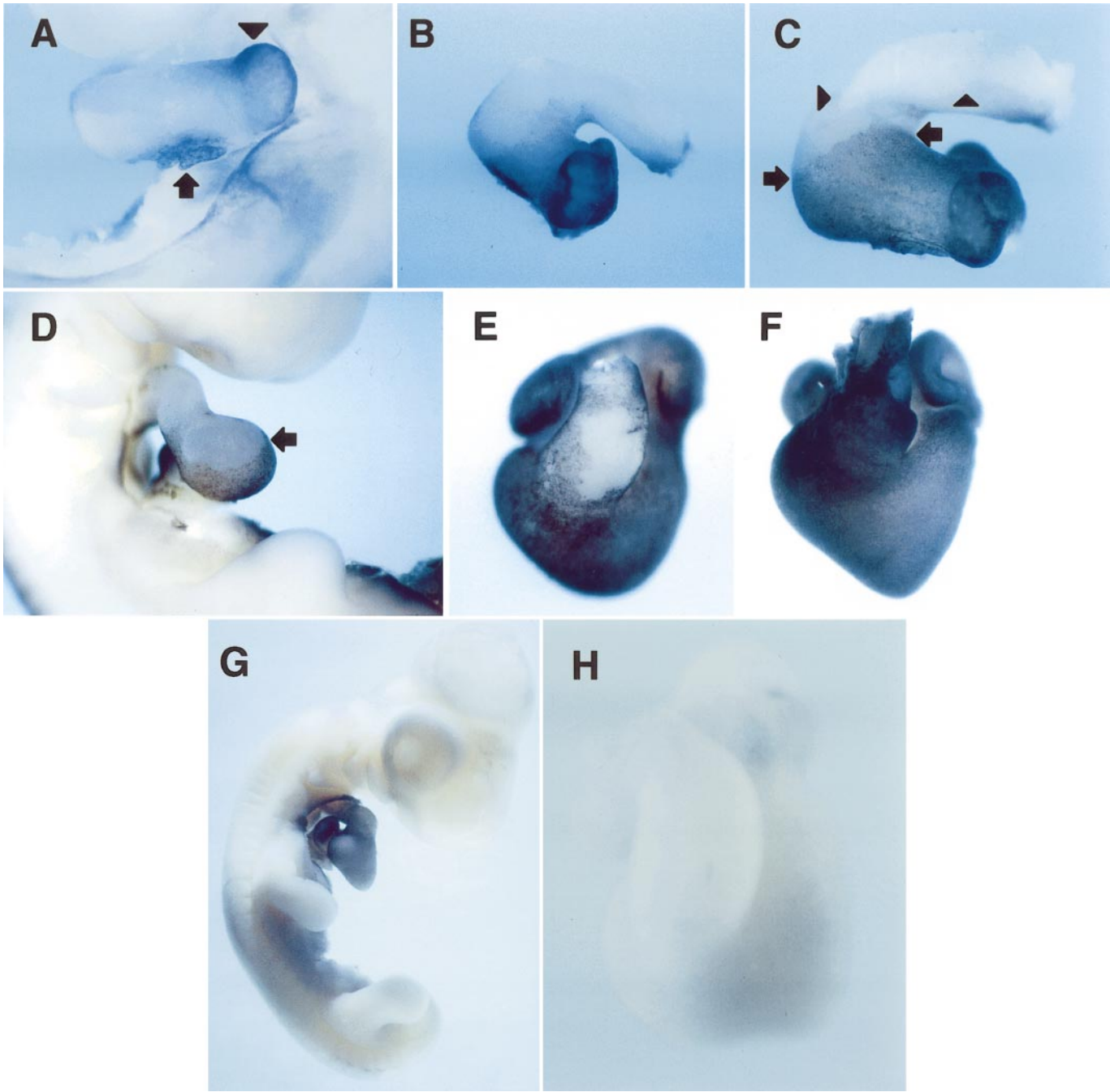

FIG. 6. RALDH2 immunoreactivity in the epicardium. (A) Stage 18 HH quail embryo. Note the presence of two distinct centers of RALDH2 expression in the epicardial organ (arrow) and in the atrium (arrowhead). (B) Stage 19 HH heart. (C) Stage 20 HH heart. Note the migration fronts of myocardial (arrowheads) and epicardial (arrows) waves of RALDH2 expression. (D) Stage 21 HH embryo. Note that the epicardial layer reaches the prospective right ventricle (arrow). (E) Stage 22 HH heart. (F) Stage 24 HH heart. (G) Stage 24 HH embryo with heart exposed after dissection of the pericardium. Note the complete enveloping of the heart by the RALDH2-expressing epicardium. (H) Stage 22 HH negative control heart. The apparent blue staining in the ventricle represents trapping of the dye. (I) RALDH2 staining of a coronal section of a stage 24 quail. Note staining in the pericardium (pc) and epicardium (ec) and the absence of RALDH2 immunoreactivity in the myocardium at this stage. Other abbreviations: a, atrium; cn, cushion; tr, trabecular tissue; v, ventricular lumen.

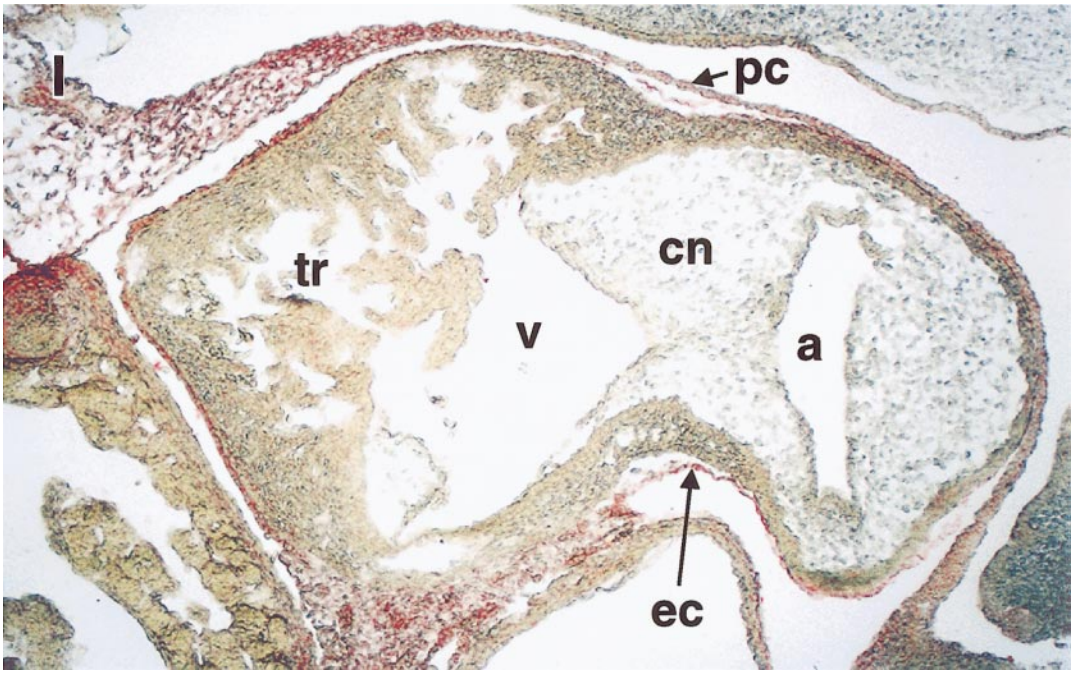

FIG. 6—*Continued*

RA signaling in the ventricle is also consistent with recent studies showing that conditional inactivation of the RXRα gene in cardiac myocytes is innocuous, while universal ablation leads to a hypoplastic phenotype (Chen *et al.*, 1998; Tran and Sucov, 1998).

These studies suggest a model whereby RA produced by RALDH2 in the epicardial layer is critical for its contribution to the developing heart, either directly by promoting expansion of the underlying ventricular myocardium or indirectly by autocrine action on the epicardium itself, resulting in secretion of other trophic factors. In addition, migratory epicardium-derived cells may persist as an embryonic fibroblast population with a role in the formation of the fibrous heart skeleton and may be the source of fibroblasts that contribute to the interstitial connective tissue of the mature heart (Grittenberger-de Groot, 1997). It will be interesting to determine to what extent these migratory cells maintain RALDH2 expression. RA signaling may also participate in the invasion of epicardial cells contributing to coronary vessel walls and atrioventricular cushions. Notably, diminished growth of atrioventricular cushion tissue was observed in RA-treated chicken embryos (Bouman *et al.*, 1998). This suggests that the putative role played by RA-mediated epicardial signaling in cushion formation is likely to be indirect, since ectopic administration of RA directly to the developing heart disrupts the proper development of cushion tissue.

In summary, the general conservation of RALDH2 synthetic patterns between mammals and avians suggests that ligand signaling in the developing heart is controlled by a common developmental strategy, namely, localized action of RA by regulated RALDH2 activation. Future analysis of the molecular mechanisms controlling dynamic patterns of RALDH2 synthesis will provide powerful points of entry to the dissection of early events in cardiac chamber specification and to the contribution of epicardial RA signaling to underlying ventricular myocardial maturation.

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