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A retinoic acid-inducible transgenic marker of sino-atrial development in the mouse heart

José Xavier-Neto[1,2], Craig M. Neville[1], Michael D. Shapiro[1,3], Leslie Houghton[1], Gang Feng Wang[4], William Nikovits Jr[4], Frank E. Stockdale[4] and Nadia Rosenthal[1,5,*]

[1]Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, MA 02129, USA
[2]Laboratório de Biologia Molecular, Instituto do Coração (InCor) Faculdade de Medicina da Universidade de São Paulo (USP) 05403-000, Brazil
[3]Museum of Comparative Zoology and Dept of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA
[4]Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305-5306, USA
[5]Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA
*Author for correspondence (e-mail: Rosentha@helix.mgh.harvard.edu)

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SUMMARY

To study the specification of inflow structures in the heart we generated transgenic animals harboring the human alkaline phosphatase (HAP) gene driven by the proximal 840 bp of a quail SMyHC3 promoter. In transgenic mice, the SMyHC3-HAP reporter was expressed in posterior heart precursors at 8.25 dpc, in sinus venosa and in the atrium at 8.5 and 9.0 dpc, and in the atria from 10.5 dpc onwards. SMyHC3-HAP transgene expression overlapped synthesis and endogenous response to retinoic acid (RA) in the heart, as determined by antibodies directed against a key RA synthetic enzyme and by staining of RARE*hsplacZ* transgenic animals. A single pulse of all-trans RA administered to pregnant mice at 7.5, but not after 8.5, dpc induced cardiac dismorphology, ranging from complete absence of outflow tract and ventricles to hearts with reduced ventricles expressing both SMyHC3-HAP and ventricular markers. Blockade of RA synthesis with disulfiram inhibited RA-induced transcription and produced hearts lacking the atrial chamber. This study defines a novel marker for atrial-restricted transcription in the developing mouse heart. It also suggests that atrial-specific gene expression is controlled by localized synthesis of RA, and that exclusion of RA from ventricular precursors is essential for correct specification of the ventricles.

Key words: Mouse embryo, Heart, Sinus venosa, Atrium, Ventricle, Retinoic acid

INTRODUCTION

Although the early stages of commitment to the cardiac phenotype have been extensively characterized (Montgomery et al., 1994), very little is known about the processes that generate cell diversity in myocardial precursors. Establishment of anterior-posterior polarity is one of the earliest decisions in cardiogenesis, and critical to the development of the incipient circulatory system. Adequate specification of inflow (posterior) and outflow (anterior) structures ensures that the venous system connects to sino-atrial myocardium displaying the highest beating rate and that the arterial system couples to the outflow tract exhibiting the lowest beating rate. This inflow-to-outflow gradient of excitability acts together with slow conducting velocities of sinus venosa, atrioventricular canal and conotruncus to generate a directional peristaltic pumping and an effective early cardiac output, despite the absence of septa, valves, papillary muscles and specialized conduction systems (De Jong et al., 1992; Moorman and Lamers, 1994).

Fate-mapping studies in chicken suggest that the inflow-outflow patterning of the vertebrate heart is already encoded in the topological organization of cells in the cardiogenic epiblast, primitive streak and cardiogenic mesoderm (Rosenquist and De Haan, 1966; Garcia-Martinez and Schoenwolf, 1993). Epiblast cells in the vicinity of the primitive streak are found later in the aorta and in the pulmonary arteries. Conversely, the most lateral cells in the cardiogenic epiblast are found later in atria and sinus venosa, while cells at an intermediate position in the cardiogenic epiblast are more likely to be found in ventricles. In the cardiogenic section of the primitive streak, rostral sections are destined to form aorta and pulmonary arteries, while progressively caudal sections are more likely to populate ventricles, atria and sinus venosa, respectively (Garcia-Martinez and Schoenwolf, 1993). Fate mapping, however, indicates neither when nor how topological information translates into specification of cell fate. Instead, analyses of the mechanisms involved in inflow-outflow specification in the vertebrate heart have relied on the use of cell-restricted markers (Yutzey et al., 1994; Yutzey and Bader, 1995).

Expression of various myocardial gene markers in different species has prompted two alternative hypotheses concerning the timing of inflow-outflow specification. According to the first hypothesis, inflow-outflow specification is achieved only after cardiac looping, when cardiac chambers are forming. This view is supported by recent data showing ubiquitous expression of the slow myosin heavy chain 3 gene (SMyHC3) in the unlooped quail heart tube, and atrial-restricted expression by the time heart chambers are being formed (Wang et al., 1996, 1998). Similar results have also been observed in mice and in chicken embryos, where atrial-specific α myosin heavy chain (MyHC) and ventricular-specific β MyHC are initially coexpressed in the primordial heart tube and become restricted later as the chambers are formed (De Groot et al., 1989; De Jong et al., 1990; Lyons et al., 1990).

According to the second hypothesis, inflow-outflow specification is already underway before looping and formation of cardiac chambers. This idea is supported by the expression patterns of chamber-specific markers in several species. In chicken, atrial-specific myosin heavy chain (AMHC1) is detected in the inflow region of the heart from stage 9+ onwards, well before any evidence of chamber formation (Yutzey et al., 1994). In mice, the ventricular-specific myosin light chain 2 (MLC2-V) promoter is activated exclusively in the anterior myocardium of the unlooped heart (Ross et al., 1996). Finally, in zebrafish, inflow structures are specifically recognized by the sino-atrial specific monoclonal antibody S46 long before atrial and ventricular chambers are individualized (Stainier and Fishman, 1992).

While the exact timing of inflow-outflow specification is still controversial, the mechanisms underlying it are even more obscure. There is however, increasing evidence that signaling by nuclear receptor pathways is required to specify sino-atrial tissue during development. In quail embryos retinoic acid (RA) insufficiency produced either by vitamin A deprivation, or by administration of antibodies against RA, leads to suppression of inflow structures of the heart (Heine et al., 1985; Twal et al., 1995). Conversely, RA excess during cardiogenesis leads to expansion of sino-atrial compartment at the expense of ventricular and outflow tissues in chicken (Osmond et al., 1991; Yutzey et al., 1994) and in zebrafish (Stainier and Fishman, 1992).

Currently, treatment with RA is the only method available to interfere with inflow-outflow specification, but since the effects of exogenous RA do not necessarily elucidate physiological processes, the linkage between retinoid signaling in the heart and inflow-outflow specification has been largely unexplored. We have recently examined RA signaling during cardiac development and described a striking correlation between domains of endogenous response to RA and immunoreactivity to a retinaldehyde-oxidizing dehydrogenase (RALDH2) that catalyzes the last step in the RA biosynthetic pathway (Zhao et al., 1996). In the mouse, RALDH2 expression colocalized with endogenous response to RA in sino-atrial tissues from 8.25 to 12.5 days post coitum (dpc), while ventricular tissue remained devoid of RA metabolism up to 12.5 dpc (Moss et al., 1998). Thus, RALDH2 expression is a reliable readout of RA synthesis in the developing heart, and its early restriction to sino-atrial structures suggests that RA signaling is involved in the specification of cardiac inflow structures (Moss et al., 1998).

Recently, Wang et al. (1996) have shown that a 168 bp fragment from the SMyHC3 promoter, containing response elements to both RA and vitamin D receptors, drives preferential atrial expression in chicken embryos when linked to an heterologous promoter in a retroviral vector. Moreover, this fragment binds both RA and vitamin D receptors (RARs and VDRs) and inhibits expression of a reporter gene in cultured ventricular, but not atrial cardiomyocytes (Wang et al., 1998). Inhibition of ventricular expression in the SMyHC3 promoter is released by mutation of an RAR/VDR binding site, suggesting that atrial specificity of the SMyHC3 gene in the quail is due to active suppression of ventricular expression.

The unique characteristics of the SMyHC3 promoter (Wang et al., 1996) provided an opportunity to assess the relationship between RA signaling and the specification of cardiac inflow structures. To establish whether expression from the quail promoter would reflect the early sino-atrial restriction of RA metabolism in a murine background, we used the proximal 840 bp of the SMyHC3 promoter to drive expression of the human alkaline phosphatase reporter gene (HAP) in transgenic mice. The expression patterns of HAP during heart development indicate that inflow specification in the mouse heart is well underway before cardiac chambers are formed and that the SMyHC3-HAP transgene is activated only in sino-atrial structures displaying active RA signaling. Perturbation of cardiac morphogenesis by inhibition of RA synthesis was accompanied by deletion of the common atrium and by reduced cardiac expression of the SMyHC3-HAP transgene, while treatment with exogenous RA was accompanied by increased HAP expression, expansion of the sino-atrial compartment, and ectopic SMyHC3-HAP expression in the ventricles. These results indicate that the SMyHC3 promoter is a target for RA.

The SMyHC3-HAP transgene is the first genetic marker restricted to sino-atrial tissues from the earliest stages of mammalian cardiogenesis through adulthood. As such, it is a useful tool to investigate the role of candidate genes in congenital atrial pathology, as well as an entry point to dissect the molecular circuitry underlying sino-atrial specification.

MATERIALS AND METHODS

SMyHC3-HAP transgenic mice

The HAP reporter gene was substituted for the chloramphenicol acetyl transferase gene in the 840DCAT containing the wild-type proximal 840 bp quail SMyHC3 promoter (Wang et al., 1996, 1998). The reporter construct was excised with *Hin*dIII and *Bam*HI, gel purified, and injected into the pronuclei of fertilized mouse eggs as described (Hogan et al., 1986). Two-cell FVB embryos were transferred to the uterus of pseudopregnant ICR females and founders were identified by PCR with primers designed to amplify a fragment of the HAP gene (C. M. Neville et al., unpublished results). Two 840-HAP transgenic lines were established. RARE*hsplacZ* transgenic embryos have been previously described (Rossant et al., 1991). Transgenic embryos were obtained from crosses between heterozygous HAP and homozygous RARE*hsplacZ* transgenic males and wild-type FVB females. Noon of the day of vaginal plug was designated as 0.5 dpc.

HAP, lacZ, antibody and in situ hybridization staining

Embryos and hearts from 7.5-15.5 dpc were collected, analyzed for expression of HAP, *lacZ* or subjected to immunohistochemistry. For HAP staining, embryos were dissected and fixed overnight at

4°C in phosphate buffered saline, pH 7.4 (PBS) containing 4% paraformaldehyde and 2 mM $MgCl_2$. Subsequently, the embryos were washed 5 times in PBS containing 2 mM $MgCl_2$, heated at 65°C for 90 minutes, washed in XP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 2 mM levamisole) and stained in XP buffer containing BCIP/NBT. β-galactosidase (β-gal) staining was performed using the X-gal substrate as described by Cheng et al. (1993). For whole-mount immunohistochemical analysis (Dent et al., 1989), rabbit polyclonal antibodies raised against RALDH2 and MLC2-V were used at dilutions of 1:200 and 1:50, respectively. Secondary antibodies were horseradish peroxidase-labeled donkey anti-rabbit IgG (Amersham NA 934), Cy3-labeled goat anti-rabbit IgG (Amersham PA 43004), both used at a 1:1000 dilution, and an anti-rabbit IgG fluorescein conjugate (Boehringer Manheim 1814257) used at 1:700 dilution. Stains were performed with DAB as a substrate, which gave RALDH2- and MLC2-V-positive tissues a brown color. For double stainings, embryos and dissected hearts were first stained for HAP or β-gal. Subsequently, embryos were fixed in Dent's fixative and submitted to immunostaining. For RALDH2/MLC2-V double immunohistochemistry, hearts were first stained using the RALDH2 antibody, fixed again in Dent's and then stained with the MLC2-V antibody and an anti-rabbit IgG alkaline phosphatase conjugate. Whole-mount in situ hybridizations were performed using Nkx-2.5 and GATA 4 antisense probes (Lints et al., 1993; Molkentin et al., 1997) according to established protocols (Wilkinson, 1992). Nkx-2.5 embryos were then fixed in Dent's and stained for RALDH2. Paraffin sections were generated by standard methods (Sassoon and Rosenthal, 1993) on previously stained hearts of 12.5 dpc embryos. Newborn and adult hearts were first sectioned and then stained for HAP with Fast Red as a substrate (C. M. Neville, unpublished results).

Retinoic acid and disulfiram treatments

All-trans RA and vitamin D_3 (calcitriol) (50 mg) were dissolved in 1 ml of ethanol, diluted to a concentration of 5 mg/ml with sunflower oil and administered (100 mg/kg) by gavage to pregnant mice at 7.5, 8.5 and 11.5 dpc (Niederreither et al., 1997). The active form of vitamin D_3, 1,25$(OH)_2$vitamin D_3, was first dissolved in DMSO, then into sunflower oil and administered at 25 μg/kg by gavage in the same manner as described above. The retinaldehyde dehydrogenase inhibitor disulfiram (1.33 mg/g) was suspended into culture medium (DMEM) and administered subcutaneously to pregnant females at noon of 6.5 dpc. Supplementary doses of disulfiram were dissolved into 40 μl of DMSO (0.33 mg/g) and administered subcutaneously at noon of 7.5 and 8.5 dpc. Control embryos received only DMEM and DMSO. Embryos were harvested at various times after treatment, their phenotypes were scored, and when appropriate, they were stained for HAP, β-gal, or subjected to immunohistochemical analysis.

Image analyses and processing

Embryos were photographed on a Nikon stereozoom dissecting microscope (SMZ-2T) and photographed with 64 ASA tungsten slide film. Whole-mount immunofluorescence and paraffin sections were photographed on a Zeiss Axiophot microscope using 400 and 100 ASA film, respectively. Images were acquired with a Polaroid Sprint Scan 35 slide scanner.

RESULTS

Atrial-specific expression of the SMyHC3-HAP transgene

For the present study, the HAP reporter gene was linked to the proximal 840 bp SMyHC3 promoter. This reporter has several advantages over the more commonly used *lacZ* reporter in that the HAP gene product is membrane bound and therefore non-diffusible, and is heat stable, providing a simple means to clear all background phosphatase activity from the tissue to be analyzed (C. M. Neville, unpublished results). In addition, the use of an alternative reporter permits simultaneous analysis of HAP and *lacZ* reporter activity in the same tissues.

Expression of the SMyHC3-HAP transgene in germline-integrated lines was localized to the heart of 9.0 dpc embryos (Fig. 1A), while nontransgenic sibs were completely devoid of HAP activity (Fig. 1B). The SMyHC3 promoter was activated in the common atria and atrioventricular canal, but not in the ventricles, which stained for MLC2-V protein (Fig. 1C). The domains of SMyHC3-HAP transgene activity and MLC2-V expression were generally mutually exclusive, except for a limited area of overlap in the atrioventricular channel (Fig. 1D).

Time course of SMyHC3-HAP expression during cardiogenesis

We analyzed the time course of cardiac SMyHC3-HAP expression from its earliest activation at 8.25 dpc to adulthood.

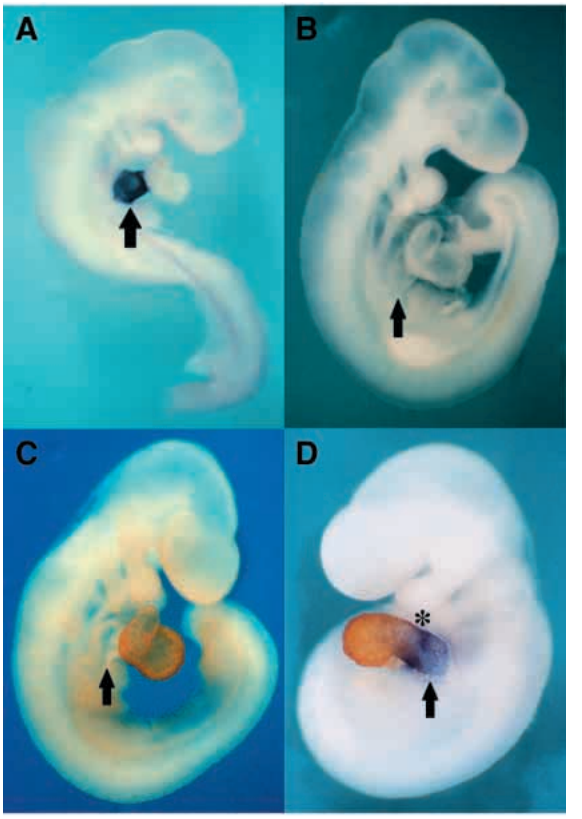

Fig. 1. The proximal 840 bp quail SMyHC3 promoter confers atrial specificity to a human alkaline phosphatase reporter gene (HAP) in the mouse. (A) HAP staining of a 9.0 dpc SMyHC3-HAP transgenic embryo. (B) HAP staining of a non-transgenic 9.0 dpc mouse embryo. (C) MLC2-V immunohistochemical staining of a 9.0 dpc SMyHC3-HAP transgenic embryo. (D) Double staining (HAP+MLC2-V) of a 9.0 dpc SMyHC3-HAP transgenic embryo. The SMyHC3-HAP transgene is expressed only in the atria (arrows) of transgenic embryos, while non-transgenic sibs do not express any HAP. The domains of SMyHC3-HAP and MLC2-V expression describe inflow (sino-atrial) and outflow compartments of the heart, respectively. Double-stained mice indicate that SMyHC3-HAP and MLC2-V overlap in the atrioventricular channel (D, *).

The transgene was first expressed in unfused heart precursors lying on the roof of the anterior intestinal portal shortly after fusion of the two heart primordia (Fig. 2A). At 8.5 dpc, transgene expression was detected in the sinus venosa and in the newly formed common atrium (Fig. 2B). Thereafter transgene expression marked the development and morphogenetic movements of sino-atrial structures, with no signs of asymmetry throughout cardiogenesis (Fig. 2C-F). The only extra-atrial transgene activity was limited to small spots of activity on the surface of the left ventricle at 11.5 dpc (Fig. 2E) and superficial staining overlying the outflow tract at 12.5 dpc (Fig. 2F). The majority of transgene expression remained essentially atrial, however (Fig. 2G).

Substrate penetration was not a limiting factor in whole heart analyses, since paraffin sections of HAP-stained hearts (Fig. 2G) yielded results comparable with cryostat sections of 15.5 dpc and adult hearts, cut on OCT medium and subsequently stained for HAP (not shown). HAP staining of paraffin sections through a newborn heart also demonstrated that the SMyHC3-HAP transgene was not expressed to any significant extent in the ventricles (Fig. 2H). Similar results were also obtained with adult hearts, (Fig. 2I), confirming that the SMyHC3-HAP transgene is expressed in sino-atrial tissues from early stages of development to adulthood.

SMyHC3-HAP expression is restricted to regions of RA synthesis and response during heart development

We have previously reported a dynamic pattern of RA synthesis and response in the developing mammalian heart (Moss et al., 1998). To determine whether the atrial specificity of the SMyHC3-HAP transgene correlated with RA signaling, we compared HAP staining with localization of the RA synthetic enzyme RALDH2, and with RA responsiveness as visualized by the RARE*hsplacZ* transgene. As previously reported, RALDH2 in 8.5 dpc embryos (Fig. 3A) overlapped endogenous response to RA (Fig. 3B) in the sinus venosa and common atrium, while the ventricles were not a site for RA metabolism. Notably, SMyHC3-HAP transgene expression was restricted to the same sino-atrial areas that displayed RA synthesis and response at 8.5 dpc (Fig. 3C) and during subsequent heart development, as seen in Fig. 3D-F.

RA acid signaling and antero-posterior patterning in the cardiac crescent

The atrial-specific patterns of RALDH2 and RARE*hsplacZ* in the early developing heart suggested that RA signaling is a

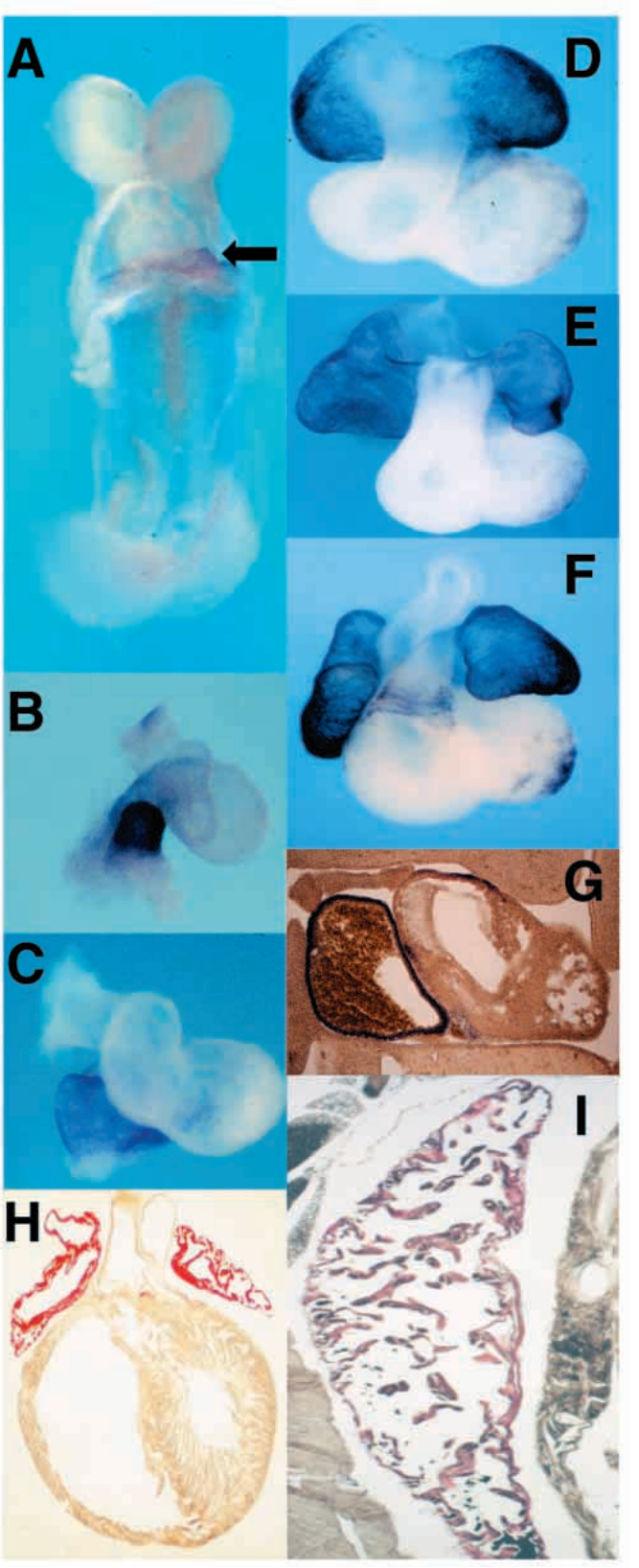

Fig. 2. Time course of SMyHC3 expression during cardiac development in the mouse. (A) 8.25 dpc embryo, (B) 8.5 dpc heart, (C) 9.5 dpc heart, (D) 10.5 dpc heart, (E) 11.5 dpc heart, (F) 12.5 dpc heart. (G) Parasagittal section of an HAP-stained 12.5 dpc heart. (H) HAP staining of a frontal section of a newborn heart. (I) HAP staining of a frontal section of an adult heart. Ventral and lateral views of hearts from 8.25-12.5 dpc embryos as well as sections of newborn and adult mice hearts indicate that the SMyHC3-HAP transgene marks sino-atrial population from the earliest stages of development to adulthood. SMyHC3-HAP expression was first observed in posterior heart precursors lying in the roof of the foregut pocket (A, arrow). SMyHC3-HAP was next observed in the sinus venosa and common atrium of 8.5 dpc embryos, in the common atrium of 9.5 dpc embryos, and in atria from 10.5 dpc embryos to adult hearts. Spotted expression was first observed at 11.5 dpc in the apex of the left ventricle and on the surface of the outflow tract at 12.5 dpc. Sectioning of newborn and adult heart showed that transgene expression did not progress in ventricles, indicating that the SMyHC3 promoter remained essentially atrial-specific.

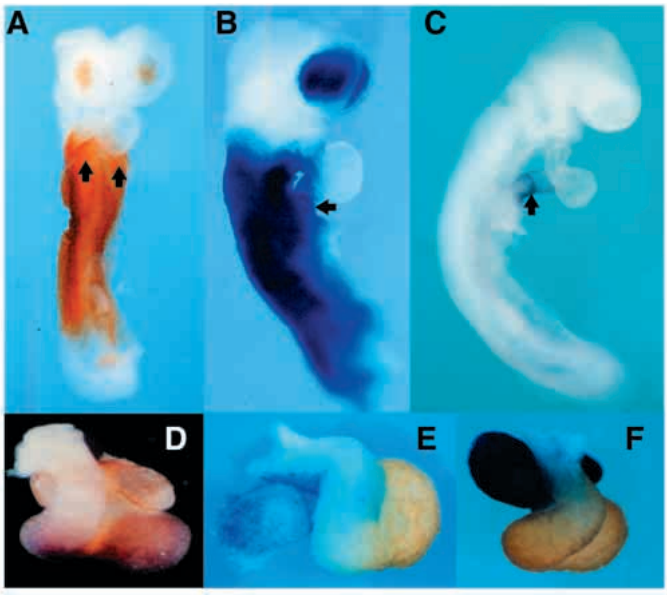

Fig. 3. SMyHC3-HAP expression colocalizes with synthesis and endogenous response to RA in the heart. (A) RALDH2 immunohistochemical staining of an 8.5 dpc embryo. (B) β-gal staining of a 8.5 dpc RARE*hsplacZ* transgenic embryo. (C) HAP staining of a 8.5 dpc SMyHC3-HAP transgenic embryo. (D) RALDH2/MLC2-V double immunohistochemical staining of a 10.5 dpc heart. (E) β-gal/MLC2-V double staining of a 10.5 dpc RARE*hsplacZ* transgenic heart. (F) HAP/MLC2-V double staining of a 10.5 dpc SMyHC3-HAP transgenic heart. In the heart RALDH2 is present in the sinus venosa (arrows) and common atrium, but is absent from the ventricle at this stage. Endogenous response to RA is also localized to sinus venosa (arrows) and is lacking in the ventricle, indicating that RALDH2 is a reliable readout of RA synthesis in the developing mouse heart. SMyHC3-HAP expression is seen in the sinus venosa and common atrium, the same structures that display both RA synthesis and response in the heart. Double-stained hearts reveal the atrial-preferential nature of both RA synthesis and endogenous response to RA against the background of ventricular-specific MLC2-V. HAP staining underscores the atrial specificity of the SMyHC3 promoter and indicates the colocalization of SMyHC3-HAP expression, RA synthesis and endogenous response to RA at this stage.

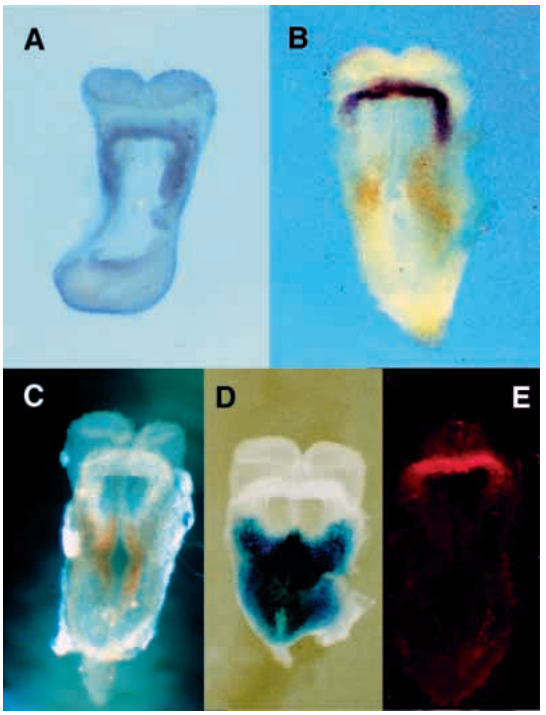

Fig. 4. The patterns of RA synthesis and response suggest that the cardiac crescent is patterned in the anterior-posterior axis by RA signaling. (A) GATA4 in situ hybridization of a 7.5 dpc embryo. (B) Double Nkx-2.5 in situ hibridization/ RALDH2 immunohistochemical staining of a 7.5 dpc embryo. (C) RALDH2 immunohistochemical staining of a 7.75 dpc embryo. (D) β-gal staining of a 7.75 dpc RARE*hsplacZ* embryo. (E) MF20 immunohistochemical staining of the embryo in C. Late gastrula mouse embryos were analyzed with antibodies against RALDH2 and stained for β-gal to determine the distribution of RA synthesis and endogenous response to RA, respectively. The distribution of differentiated and undifferentiated cardiac precursors was determined by immunohistochemistry using MF20 antibodies and by in situ hybridization with mRNA antisense probes for Nkx-2.5 and GATA4. RALDH2 immunoreactivity and β-gal staining indicate that both RA synthesis and endogenous response to RA are likely to overlap only in the most posterior cardiac precursors in the cardiac crescent as determined by the distribution of GATA4 mRNA and of Nkx-2.5 message in Nkx-2.5 /RALDH2 double stained embryos. MF20 immunoreactivity in the embryo depicted in E suggests that posterior cardiac precursors are patterned by RA signaling well before they differentiate into muscle cells.

likely candidate to impart antero-posterior information to the cardiac field. To describe the spatial relationship between RA action and the cardiogenic program, we examined the expression patterns of markers for cardiac muscle differentiation and RA signaling in late gastrula embryos. Transcripts encoding Nkx-2.5 and GATA 4 revealed the distribution of cardiac precursors in the heart field (Lints et al., 1993; Molkentin et al., 1997), while MF20 antibody directed against myosin heavy chain marked differentiated muscle. RALDH2 immunoreactivity and expression of the RARE*hsplacZ* transgene were utilized to indicate the spatial distribution of RA synthesis and endogenous response to RA, respectively. As seen in Fig. 4, only the most posterior cells expressing GATA4 or Nkx-2.5 (Fig. 4A and B, respectively) synthesized RA (Fig. 4C) or activated RA-responsive gene expression (Fig. 4D). Conversely, MF20 immunoreactivity in the most anterior, but not in posterior, heart precursors (Fig. 4E) suggested that RA signaling affects the patterning of sino-atrial structures before they express markers of muscle differentiation.

Exogenous RA induces ectopic SMyHC3-HAP transgene expression and disrupts antero-posterior specification of the heart

In early cardiac development, RA signaling is posteriorly restricted and absent from anterior ventricular precursors (Moss et al., 1998). Since SMyHC3-HAP transgene activity was similarly restricted (Figs 2A, 5A), it constituted an obvious candidate for induction by RA in the developing mouse heart. To explore this possibility, we treated 7.5, 8.5 and 11.5 dpc pregnant females with a single pulse of all-trans RA by gavage, and analyzed SMyHC3-HAP and RARE*hsplacZ* transgenic

embryos 24 hours after treatment. Fig. 5B shows that all-trans RA administered at 7.5 dpc extended the domains of SMyHC3-HAP expression anteriorly to include the fused portions of the heart by 8.5 dpc. This tissue does not normally stain for the atrial-specific SMyHC3-HAP transgene (Figs 2A and 5A) but stains positively for a ventricular-specific MLC2-V*lacZ* transgene (Ross et al., 1996). Remarkably, treatment with all-trans RA 1 or 4 days later (8.5 or 11.5 dpc) failed to activate the SMyHC3 promoter to any significant extent in the presumptive ventricles (Fig. 5C and data not shown). Ectopic activation of the SMyHC3-HAP transgene by exogenous RA was therefore restricted to a narrow window of sensitivity around 7.5 dpc.

By 8.5 dpc, endogenous RA signaling was still restricted to sino-atrial tissues (Fig. 5D). However, all-trans RA administered at either 7.5 or 8.5 dpc could activate the RARE*hsplacZ* transgene throughout the embryo (Fig. 5E and F, respectively), indicating that by this stage, anterior structures were competent to respond to RA. This indicated that other factors regulating SMyHC3-HAP expression must be responsible for delimiting its activity to the atrial compartment. It was therefore necessary to determine whether anterior expansion of SMyHC3-HAP activity by exogenous RA was an artifact of transgene expression, or whether it reflected a general respecification of ventricular precursors to an atrial identity.

To characterize the morphological impact of precocious RA

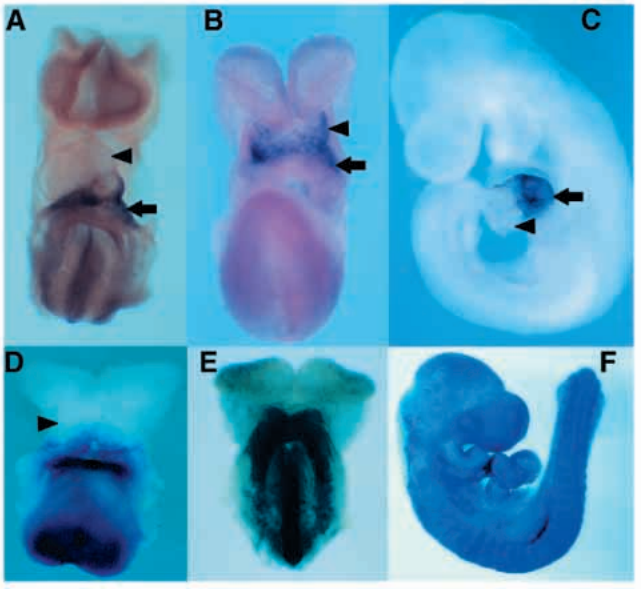

Fig. 5. RA activates ectopic expression of the atrial-specific SMyHC3-HAP transgene in ventricles. (A) HAP staining of a control 8.5 dpc SMyHC3-HAP embryo. (B) HAP staining of a 8.5 dpc SMyHC3-HAP transgenic embryo treated with RA at day 7.5 dpc. (C) HAP staining of a 9.5 dpc SMyHC3-HAP transgenic embryo treated with RA at day 8.5 dpc. (D) β-gal staining of control 8.5 dpc RARE*hsplacZ* transgenic embryo. (E) β-gal staining of a 8.5 dpc RARE*hsplacZ* transgenic embryo treated with RA at day 7.5 dpc. (F) β-gal staining of a 9.5 dpc RARE*hsplacZ* transgenic embryo treated with RA at day 8.5 dpc. RA administered at 7.5 dpc induced retarded growth and extended SMyHC3-HAP expression to include anterior heart precursors that do not normally express this transgene. Staining of RARE*hsplacZ* transgenic embryos indicated that RA-sensitive expression was induced throughout 8.5 dpc embryos, including all heart precursors, while control animals showed a clear restriction of β-gal staining to posterior heart precursors. Remarkably, RA administered at 8.5 dpc failed to activate expression of the SMyHC3-HAP transgene in the ventricles, although β-gal staining of RARE*hsplacZ* transgenics indicated that RA was fully competent to activate transcription throughout 9.5 dpc embryos. These results clearly indicate that there is a narrow window of opportunity for induction of an atrial-specific marker such as the SMyHC3-HAP transgene in the ventricles. Arrows represent the posterior limit of the heart and arrowheads indicate ventricular tissue.

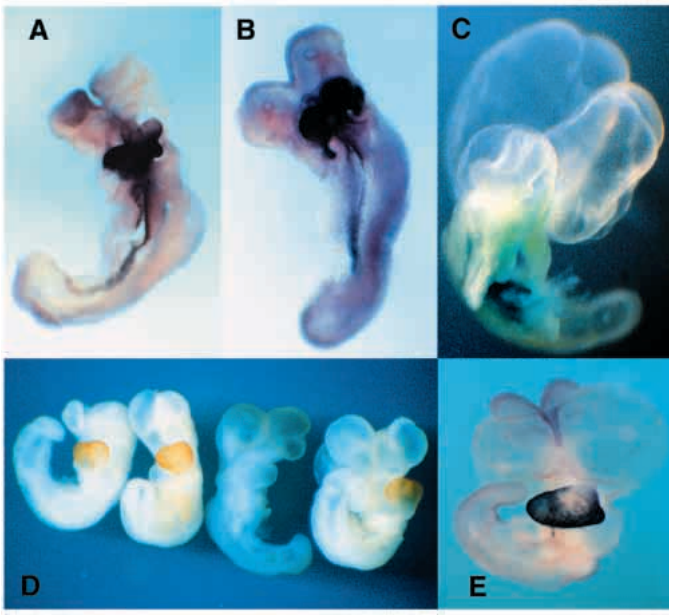

Fig. 6. RA disrupts anterior specification in the mouse heart. To determine the morphological impact of ectopic activation of the atrial program in the ventricles, pregnant females were treated with RA administered by gavage at 7.5 dpc. Their transgenic SMyHC3-HAP embryos were harvested 48 and 72 hours later and stained for HAP or for MLC2-V as required. (A,B) HAP staining of 9.5 dpc SMyHC3-HAP transgenic embryos treated with RA at day 7.5 dpc. (A) Hearts with marked atrial dominance in which the common atrium empties into a stalk resembling the atrioventricular channel, which empties into a closed chamber displaying a texture characteristic of normal ventricles. There was no macroscopic evidence of any outflow structure. HAP staining was intense in the atrial region and in the putative atrioventricular channel, but was much less intense in the putative ventricular chamber. (B) Hearts occupying a central position and showing incomplete fusion of the bilateral cardiac vesicles (cardiac bifida). In these animals HAP staining was present throughout the heart. (C) β-gal staining of 9.5 dpc RARE*hsplacZ* transgenic embryos treated with RA at day 7.5. These embryos showed the same morphological features as SMyHC3-HAP, but showed remarkable down-regulation of β-gal activity 48 hours after treatment. Note the pericardial bubble indicative of cardiac failure. (D) MLC2-V immunohistochemical staining of 10.5 dpc SMyHC3-HAP transgenic embryos treated with RA at day 7.5 dpc. Ventricular cells are highlighted 48 hours after treatment with RA. Note that the extent of ventricular preservation after RA treatment is variable, with some embryos showing very little or no ventricular tissue at all. (E) HAP staining of 10.5 dpc SMyHC3-HAP transgenic embryo treated with RA at day 7.5 dpc. HAP staining 72 hours after treatment indicates that in the most severe cases RA can induce an almost complete conversion of the heart into the atrial phenotype.

signaling in anterior heart precursors, we harvested SMyHC3-HAP and RARE*hsplacZ* transgenic embryos either 48 or 72 hours after administration of all-trans RA at 7.5 dpc. A total of 52 embryos harvested 48 hours after RA treatment displayed retarded growth and a range of cardiac phenotypes that could be grouped into distinct categories. The most frequent category (69%) displayed marked atrial dominance. In these hearts the common atrium emptied into a stalk resembling the atrioventricular channel, which in turn emptied into a closed chamber displaying a texture characteristic of normal ventricle (Fig. 6A). Notably, these hearts were still beating, with contractions arising from the atrial tissue and travelling anteriorly up to the putative ventricles. Within this category, the amount of putative ventricular tissue was varied, with some animals having almost none. There was no evidence of any outflow structure and a pericardial bubble invariably enveloped the heart. SMyHC3-HAP staining was intense in the atrial region and in the putative atrioventricular channel, but was much less intense in the putative ventricles (Fig. 6A).

In 23.6% of RA-treated embryos, the heart occupied a central position and showed incomplete fusion of the bilateral cardiac vesicles (cardiac bifida). In these animals HAP staining was present throughout the heart (Fig. 6B). Only one embryo out of 52 showed almost normal heart morphology, with well-defined atria, ventricles and conotruncus. In the latter, SMyHC3-HAP staining was present in the atria, in the atrioventricular channel and in spots over the ventricle (not shown). Remaining embryos were dead, undergoing resorption (7.4%). RARE*hsplacZ* embryos showed the same morphological features 48 hours after RA treatment. However, in contrast to the SMyHC3-HAP transgene, expression of the *lacZ* transgene was dramatically downregulated throughout hearts with atrial dominance, indicating that a pulse of RA per se is not sufficient to drive sustained ectopic expression of all RA-inducible genes. Note also the pericardial bubble indicating pericardial edema secondary to heart failure (Fig. 6C).

Embryos harvested 72 hours after RA treatment at 7.5 dpc showed essentially the same basic morphological features described above (Fig. 6D). Staining for MLC2-V confirmed that anterior structures in the heart indeed represented the remaining ventricular tissue and emphasized the variability in the extent of ventricular preservation after treatment with RA (compare with hearts in Figs 1C, 3F). In some embryos, no immunoreactivity to MLC2-V could be found in the heart, which showed no morphological signs of ventricular tissue (Fig. 6D). In a similar embryo, atrial-specific SMyHC3-HAP was expressed throughout the heart, suggesting a complete conversion to the atrial phenotype (Fig. 6E). These results establish the SMyHC3-HAP transgene as a bona fide marker of atrial identity, and further suggest that the temporally restricted perturbation of its expression pattern by exogenous RA reflects a general mechanism of atrial specification rather than an artifact of transgene expression.

In contrast to RA, neither vitamin D_3 or 1,25$(OH)_2$vitamin D_3 perturbed transgene expression in SMyHC3-HAP embryos (not shown), making it unlikely that the RARE/VDRE site represents a positively acting vitamin D-responsive element in the murine context.

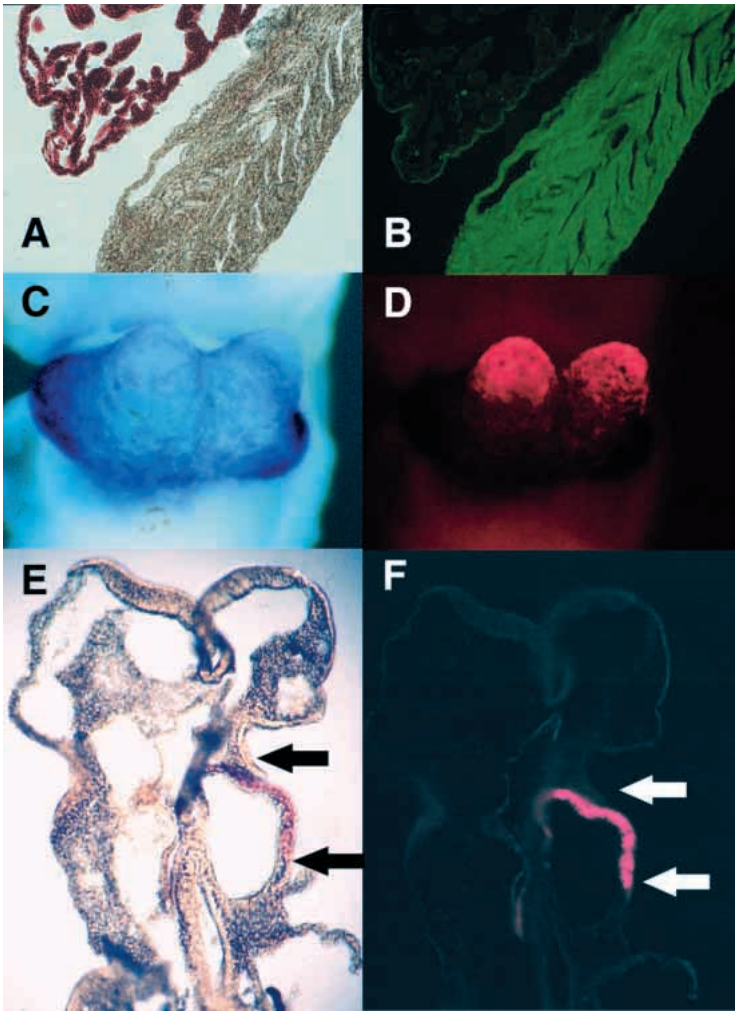

Fig. 7. Expression of SMyHC3-HAP and MLC2-V suggests complex mechanisms for induction of atrial dominance by RA. (A) HAP staining of a parasagittal section taken from a newborn SMyHC3-HAP transgenic animal. (B) MLC2-V immunohistochemical staining of the same section as in A. (C) HAP staining of a 9.5 dpc SMyHC3-HAP transgenic embryo treated with RA at 7.5 dpc. (D) MLC2-V immunohistochemical staining of the same embryo as in C. (E) HAP staining of a parasagittal section taken from a 9.5 dpc SMyHC3-HAP transgenic embryo treated with RA at 7.5 dpc. (F) MLC2-V immunohistochemical staining of the same section as in E. Pregnant females were treated with 100 mg/kg of all-trans RA dissolved in sunflower oil and administered by gavage at 7.5 dpc. Their transgenic SMyHC3-HAP siblings were harvested 48 hours later and either stained as whole mounts or embedded in paraffin and sectioned at 5 μm. Sections and whole mounts were stained for HAP using Fast Red or BCIP/NBT as substrates, respectively. MLC2-V immunoreactivity was detected in sections and whole mounts using rabbit polyclonal antibodies and anti-rabbit Cy3. Sections of newborn hearts were used to demonstrate atrial and ventricular specificity of SMyHC3-HAP and MLC2-V markers, respectively, and were stained as described above, except for the use of anti-rabbit FITC as a conjugate. HAP staining of a parasagittal section through a RA-treated embryo shows expression of the atrial-specific SMyHC3-HAP transgene throughout the antero-posterior axis of the heart in RA-treated embryos (arrows in E and F). MLC2-V immunoreactivity indicates that both ventricular-specific (MLC2-V) and atrial-specific markers are coexpressed in the hearts of RA-treated embryos.

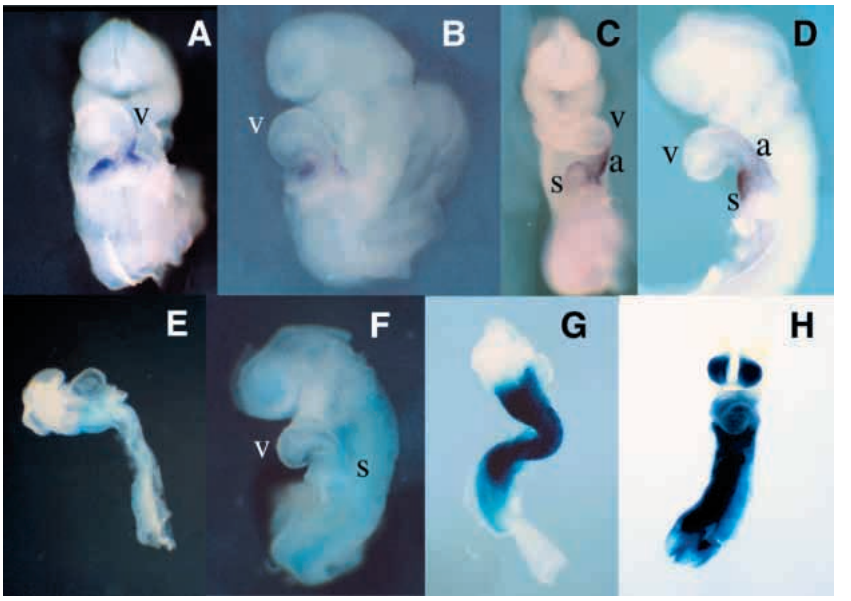

Fig. 8. Retinaldehyde dehydrogenase inhibition by disulfiram blocks development of the atrial chamber. (A) Frontal view of a disulfiram-treated 9.0 dpc SMyHC3-HAP transgenic embryo. Note the absence of organized sino-atrial structures, the overall reduction of HAP staining in the heart and the inhibition of embryonic turning. (B) Left lateral view of a disulfiram-treated 9.0 dpc SMyHC3-HAP embryo. Note the enlarged ventricular cavity, the lack of organized sino-atrial structures, the marked reduction in HAP staining and the inhibition of embryonic turning. (C) Frontal view of a control 9.0 dpc SMyHC3-HAP transgenic embryo. Note the presence of well defined sinus venosa and common atrium. (D) Left lateral view of a control 9.0 dpc SMyHC3-HAP embryo. Note the presence of distinct sinus venosa and common atrium. (E) Right lateral view of a disulfiram-treated 8.0 dpc RARE*hsplacZ* embryo. (F) Left lateral view of a disulfiram-treated 9.0 dpc RARE*hsplacZ* embryo. Note the marked reduction in *lacZ* expression associated with an enlarged ventricle, the absence of a defined common atrium and the inhibition of embryonic turning. (G) Right lateral view of a control 8.0 dpc SMyHC3-HAP transgenic embryo. (H) Frontal view of a control 8.5 dpc RARE*hsplacZ* embryo. a, common atrium, s, sinus venosa, v, common ventricle.

Coexpression of atrial and ventricular markers in RA-treated hearts

Staining of RA-treated embryos for HAP and MLC2-V indicated that in normal as well as in most RA-treated embryos, atrial and ventricular-specific markers are still expressed in separate domains (Figs 6A, 7A-D, and data not shown). Thus, most RA-treated hearts still show some degree of inflow-outflow specification. However, the possibility that atrial and ventricular markers were coexpressed in some cells suggests mechanisms by which atrial dominance is induced by RA. To investigate this possibility we searched for cells coexpressing the SMyHC3-HAP transgene and MLC2-V in the hearts of RA-treated embryos. Fig. 7E depicts a parasagittal section through a 9.5 dpc SMyHC3-HAP transgenic embryo treated with RA 48 hours earlier (at 7.5 dpc). Expression of the atrial-specific SMyHC3-HAP transgene extended throughout the antero-posterior axis of the heart (Fig. 7E). Notably, the same cells also expressed the ventricular-specific marker MLC2-V (Fig. 7F). The presence of cells coexpressing inflow and outflow markers suggest that although RA at high doses could act by deletion of ventricular precursors (Osmond et al., 1991; Stainier and Fishman, 1992; Yutzey et al., 1994), it may also act by respecifying outflow precursors to inflow cell fates (Yutzey et al., 1995), or by expansion of a population of cells already expressing both markers (see Fig. 1D).

RA synthesis inhibition by disulfiram abolishes RA-induced transcription and blocks the development of the atrial chamber

To examine the morphological consequences of reduced RA availability on antero-posterior patterning of the mouse heart we treated transgenic SMyHC3-HAP and RARE*hsplacZ* with the retinaldehyde dehydrogenase inhibitor disulfiram (Stratford et al., 1996; Costaridis et al., 1996). As depicted in Fig. 8A,B, disulfiram produced mouse embryos lacking organized sino-atrial structures. These disulfiram-treated animals displayed enlarged ventricles that slowly tapered off into a rudimentary sinus venosa. HAP staining confirmed the lack of an organized common atrial chamber, with only limited staining being present anterior to the anterior intestinal portal (Fig. 8A,B, compare with controls in Fig. 8C,D). β-gal staining of RARE*hsplacZ* embryos (Fig. 8E,F) revealed an almost complete inhibition of*LacZ* expression when compared to normal controls (Fig. 8G,H).

The persistence of HAP-expressing cells in these disulfiram-treated embryos could reflect a developmental delay in atrial specification. Alternatively the levels of RA required to activate the SMyHC3 may be slightly lower than the ones required for RARE hsp-lacZ expression, such that residual RA signaling could still specify a few cells to a posterior fate. Moreover, disulfiram may have lowered RA levels below a critical threshold only after a limited group of cells had already committed to the sino-atrial phenotype, Finally, cardiac precursors fated to give rise to atrial tissues might be more sensitive to RA depletion than are those which give rise to the sinus venosa. Nevertheless, the fact that these disulfiram-treated embryos die at 10.5 dpc without ever developing an atrial chamber supports a critical role for RA in atrial specification.

DISCUSSION

The expression patterns of transgenes driven by tissue-restricted DNA regulatory elements can provide an important entry point for studying the molecular pathways involved in organogenesis. The utility of such markers for the study of early embryonic events depends on correct timing and spatial distribution of transgene expression. It is also crucial that transgene activation be tightly associated with the developmental program under study, so that perturbations in transgene expression profiles accurately reflect the extent to

which the organ or tissue in question is affected by the experimental manipulation.

The SMyHC3-HAP transgene described in this study satisfies both criteria. First, the SMyHC3 promoter currently represents the only cis-regulatory element marking the atrial population from the earliest stages of murine cardiogenesis to adulthood. The activation of this promoter contrasts with other previously described sino-atrial markers, which are first expressed in both atria and ventricles and then become atrially restricted only at late stages of development or during the perinatal period (Sweeney et al., 1987; Zeller et al., 1987; De Groot et al., 1989; De Jong et al., 1990; Lyons et al., 1990). Second, SMyHC3-HAP expression is a faithful marker of the atrial phenotype in normal hearts and in hearts where either atrial (Fig. 6) or ventricular (Fig. 8) dominance is induced.

SMyHC3-HAP expression also revealed that cardiac precursors are already patterned along the AP axis. The caudalmost population sequentially expresses RALDH2, RARE*hsplacZ* and SMyHC3-HAP in response to activation of RA signaling, a pathway previously shown to be involved in antero-posterior patterning of avian and fish hearts (Heine et al., 1985; Twal et al., 1995; Osmond et al., 1991; Stainier and Fishman, 1992; Yutzey et al., 1994).

Together with previous studies, these data establish the SMyHC3 promoter as a target for the genetic program specifying sino-atrial identities in the primitive heart. Further dissection of cis-regulatory elements responsible for its spatial and temporal control will shed light on the inductive mechanisms underlying antero-posterior patterning in the mammalian myocardium. The properties of the SMyHC3 promoter also make it a novel experimental tool for targeting expression of transgenes exclusively to atrial cells in the developing and mature heart.

The SMyHC3-HAP transgene marks precursors of inflow structures

Expression of the SMyHC3-HAP transgene is initiated in unfused posterior cardiac precursors lying on the roof of the anterior intestinal portal (Fig. 2A). These cells constitute the caudalmost population of cardiac precursors, as defined by the expression domains of Nkx-2.5 as well as GATA 4 (Lints et al., 1993; Molkentin et al., 1997; J. Xavier-Neto, M. D. Shapiro and N. Rosenthal, unpublished observations). The early activation of the SMyHC3 promoter in posterior heart precursors, and its subsequent confinement to atrial muscle at all stages of development, suggests that the SMyHC3-HAP transgene marks a lineage of sino-atrial precursors. Alternatively, the SMyHC3-HAP transgene could be sequentially activated in posterior heart precursors, then in the sinus venosa and common atrium, and finally in the pulmonary vein bud that grows to encompass the primitive left atrium and to form most of the mature left atrial tissue (Moore, 1992). As the fate of posterior precursors in the mouse has yet to be determined, we cannot formally exclude the latter hypothesis. However, it is likely that murine sino-atrial precursors reside in the roof of the anterior intestinal portal, as do their chicken counterparts (Rosenquist and De Haan, 1966).

RA and SMyHC3-HAP transgene expression

During cardiogenesis HAP expression patterns indicate that cardiac precursors are already patterned along the AP axis well before chamber formation. The caudalmost population expresses the SMyHC3-HAP transgene shortly after activation of RALDH2 and RARE*hsplacZ* (Fig. 2A). The present study confirms that the SMyHC3-HAP transgene is responsive to RA. This may be a direct mechanism involving RAREs in the SMyHC3 promoter; however, it is equally possible that transgene expression is mediated downstream of RA, by activation of a genetic pathway of atrial specification.

The patterns of expression of SMyHC3-HAP and RARE*hsplacZ* transgenes shed some light on these possibilities. As shown in Fig. 6, exogenous RA treatment produced embryos with hearts displaying marked atrial predominance, with no discernible conotruncus and with very little ventricular tissue as defined by immunoreactivity for the ventricular-specific protein MLC2-V. In the most severely affected embryos RA induced the appearance of hearts completely dominated by the atrial phenotype, as characterized by absence of immunoreactivity for MLC2-V. In all cases expression of the SMyHC3-HAP transgene closely correlated with RA-induced atrial respecification (Fig. 6). However, in contrast to the SMyHC3-HAP transgene the RARE*hsplacZ* transgene failed to sustain cardiac expression 48 hours after RA treatment (Fig. 6C). This indicates that a pulse of exogenous RA is not sufficient to maintain ectopic expression of the RARE*hsplacZ* marker, leaving open the possibility that continued activation of the SMyHC3 promoter is a secondary consequence of atrial induction.

Antero-posterior patterning of the heart field by RA signaling

At late gastrulation (7.5 dpc) the cardiac field is already established and expresses markers of commitment such as Nkx-2.5 and GATA 4. The distribution of RA-signaling markers at this stage suggests that only posterior heart precursors will receive information from RA, before they express differentiation markers (see Fig. 4). RA signaling may therefore be acting to confer antero-posterior information to the cardiac field and, possibly, repressing terminal cardiac muscle differentiation until the atrial program can be properly elaborated. This speculation is supported by later roles of RA in retarding differentiation of ventricular cells in the compact zone as demonstrated in vitamin A-deficient embryos, RA receptor knockout lines (Wilson and Warkany, 1949; Sucov et al., 1994; Kastner et al., 1997) as well as in the heart field of *Xenopus* embryos (Drysdale et al., 1997).

Examination of the expression patterns of the SMyHC3-HAP transgene and the endogenous MLC2-V gene sheds light on the mechanisms that generate diversity along the antero-posterior axis of the developing mouse heart. As shown in Fig. 3F, these genes are respectively expressed in sino-atrial and ventricular domains (Ross et al., 1996), reflecting an inflow-outflow rather than a chamber-specific distribution. This suggests that inflow-outflow programs of genetic expression constitute the primary blueprints of antero-posterior organization in the mouse heart. The superimposition of SMyHC3-HAP and MLC2-V gene expression in the atrioventricular canal indicates that the genetic programs specifying inflow and outflow compartments intersect at this region, which may serve to pattern structures emerging from the atrioventricular boundary.

Timing of atrial specification and commitment

The timing of exposure has always been considered a critical determinant in the teratogenic effects of RA (Shenefelt et al., 1972). This view is confirmed by the data in this study, showing that the susceptibility of the embryonic heart to perturbation by ectopic RA is limited to a specific developmental timeframe. A narrow window of opportunity exists for atrial respecification of ventricular precursors around 7.5 dpc, reflecting a period during which inflow-outflow fates in the heart remain flexible and responsive to extrinsic cues. Thus cardiac cells, while they still occupy the cardiac crescent, become patterned in the antero-posterior axis and specified to inflow and outflow identities.

The absence of any significant expansion in the domains of SMyHC3-HAP expression in the hearts of embryos treated with RA at 8.5 dpc. indicates that the capacity for ventricular cell respecification is lost by the time cardiac compartments display clear anatomical separation. By this stage, cardiac tissue has committed to inflow or outflow fates and the genetic program specifying sino-atrial morphogenesis is either inaccessible to perturbation by RA, or actively repressed in differentiated ventricular and outflow compartments.

Cis-regulatory circuitry of atrial specification

A comparison between the expression patterns of the SMyHC3-HAP transgene in mice and the endogenous SMyHC3 gene in quail indicates that the SMyHC3 promoter is responding to different positional cues in the two systems. In mouse embryos, the SMyHC3-HAP transgene is not expressed in the developing ventricles to any significant extent, remaining sino-atrial throughout adulthood. In quail embryos, both anterior (ventricular and outflow) and posterior (sino-atrial) cardiac precursors activate the endogenous SMyHC3 promoter at early stages of development. As the quail heart develops and forms cardiac chambers, the SMyHC3 gene is gradually downregulated in the ventricles to become atrial-specific by embryonic day 3.0 (Wang et al., 1998).

In the quail the RARE/VDRE site functions by restricting SMyHC3 promoter activity anteriorly, such that a promoter bearing mutation in the RARE/VDRE site was expressed in ventricular avian cell cultures (Wang et al., 1996, 1998). Whether this site plays a similar inhibitory role in the developing outflow structures of the mouse heart remains to be established. However it is unlikely that the RARE/VDRE site might instead comprise a positively acting vitamin D-responsive element in the murine context, since transient treatment of pregnant SMyHC3-HAP transgenic mice with vitamin D_3 or 1,25$(OH)_2$vitamin D_3 did not perturb transgene expression in SMyHC3-HAP embryos (not shown). The RARE/VDRE site contained in the168 bp fragment from the SMyHC3 promoter is a natural candidate to mediate RA responsiveness of the SMyHC3-HAP transgene. However, other cis-regulatory elements, lying within the 840bp SMyHC3 promoter, might instead comprise direct targets for RA, or alternatively, the action of RA on the SMyHC3 promoter activity in the atria is indirect through intermediate regulatory pathways. We are currently exploring these possibilities.

A model for sino-atrial specification

We have recently proposed a model for specification of cardiac inflow structures in mammals (Moss et al., 1998). According to the model, the inflow compartment of the heart is defined by the capacity to synthesize RA and activate transcription of RA-sensitive genes. Regulation of the atrial-specific SMyHC3-HAP transgene by RA described in the present study lends further support to the model, and suggests that specification of the inflow compartment requires interaction between pathways specifying cardiac tissue and antero-posterior polarity. In this two-step model the distribution of cardiac-restricted factors marks committed cardiac precursors while RA, directly or indirectly, imparts to these cells a posterior fate. The interplay between cardiac regulators and RA signaling presumably shapes posterior heart precursors into sinus venosa and atria, while the absence of RA, cardiac restricted factors allows anterior precursors to differentiate into ventricular and outflow compartments.

This model is consistent with data described here and with previous work by a number of laboratories. For instance, inhibition of RA signaling would be predicted to allow cardiac precursors to differentiate as ventricular or outflow tract cells and produce hearts missing inflow structures, while extension of RA signaling would induce the sino-atrial genetic program in all cardiac precursors and produce hearts lacking ventricular and outflow structures. These outcomes of RA excess and insufficiency have indeed been observed in quail, chicken and zebrafish (Heine et al., 1985; Twal et al., 1995; Osmond et al., 1990; Stainier and Fishman, 1992) and first described here in a mammalian system (Figs 6, 7 and 8). It is predicted that targeted disruption of genes involved in the RA biosynthetic pathway will have important consequences for the architecture and function of the cardiovascular system. Further dissection of the SMyHC3 promoter in the context of these models will undoubtedly open up mechanisms of cardiac antero-posterior specification to molecular scrutiny.

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Note added in proof

Recently, Niederrheiter et al. (*Nature Genetics* **21**, 444-448, 1999), have shown that disruption of the RALDH2 gene results in embryonic lethality and that RALDH2 knockout mice do not develop an atrial chamber. These results are in full agreement with our model of atrial specification and indicate the pivotal role played by retinoic acid in antero-posterior patterning of the heart.

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