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Expression of Retinol Binding Protein and Transthyretin During Early Embryogenesis

MATTHEW BARRON,[1] DONNA MCALLISTER,[1] SUSAN M. SMITH,[2] AND JOHN LOUGH[1]*
[1]*Department of Cell Biology, Neurobiology, and Anatomy and Cardiovascular Research Center, Medical College of Wisconsin, Milwaukee, Wisconsin*
[2]*Department of Nutritional Sciences, University of Wisconsin-Madison, Madison, Wisconsin*

ABSTRACT **Previous studies have shown that anterior lateral plate endoderm from stage 6 chicken embryos is necessary and sufficient to enable precardiac mesoderm to complete its cardiogenic program in vitro, culminating in a rhythmically contractile multicellular vesicle (Sugi and Lough [1994] Dev. Dyn. 200:155–162). To identify cardiogenic factors, we have begun to characterize proteins that are secreted by endoderm cell explants. Fluorography of proteins from endoderm-conditioned medium revealed 1–2 dozen bands, the most prominent of which migrated at approximately 17 and 25 kD. The bulk of the 17-kD band, which migrates near FGFs and subunits of the transforming growth factor-β family, was identified by N-terminal sequencing as transthyretin (TTR). A component of the 25-kD band was identified by Western blotting as retinol binding protein (RBP). RT/PCR analysis revealed that mRNAs for both proteins are in the embryo as early as stage 3. In situ hybridization localized these mRNAs to the extraembryonic endoderm at stage 6, after which they were detected in endoderm overlying the embryo proper, including the developing heart. Later, RBP and TTR mRNA and protein were detected in cells associated with the developing heart. Western blotting of whole embryo proteins revealed the presence of RBP by stage 7, followed by sequential increases to stage 25; by contrast, content of RBP in isolated hearts peaked at stage 14, then declined. Immunohistochemistry revealed the presence of RBP protein in the extracellular matrix subjacent to lateral plate endoderm beginning at stage 8; upon formation of the definitive heart, intense staining was observed in the cardiac "jelly." By contrast TTR was intracellular, first detected as subtle deposits in stage 6 embryonic endoderm, which by stage 8 were prominent in the dorsally invaginated endoderm subjacent to the precardiac splanchnic mesoderm. At stages 11–14, TTR was detected only in myocardial cells. Such localization of RBP and TTR may indicate a role in the transport and distribution of retinol and thyroid hormone, respectively, from yolk to embryo prior to establishment of the circulatory system, and is suggestive of a subsequent role in heart development.** ***Dev. Dyn. 1998;212:413–422.*** **© 1998 Wiley-Liss, Inc.**

Key words: anterior lateral plate endoderm; heart forming region (HFR); cardiogenesis; immunohistochemistry; in situ hybridization; precardiac mesoderm; transthyretin (TTR); retinol binding protein (RBP); Western blotting

INTRODUCTION

Using a cell culture system that recapitulates cardiogenesis, we previously demonstrated that anterior lateral endoderm in the heart-forming region (HFR) of the stage-6 chick embryo is necessary and sufficient to support terminal differentiation in precardiac mesoderm cells (Sugi and Lough, 1994). Consequently, a major objective of this laboratory has been to characterize secretory products of AL endoderm that may have a role in the cardiogenic process. We have accordingly shown that FGF-2 and activin-A, both of which are associated with anterior lateral endoderm cells (Kokan-Moore et al., 1991; Parlow et al., 1991), can mimic the cardiogenic effect of endoderm on precardiac mesoderm cells in vitro (Sugi and Lough, 1995). More recently it was demonstrated that FGF isoproteins 1 and 4, which are also localized in anterior lateral plate endoderm, exhibit similar cardiogenic activity (Zhu et al., 1996). Although bone morphogenetic protein-2 (BMP-2) is also expressed by anterior lateral endoderm (Lough et al., 1996; Schultheiss et al., 1997), this growth factor is unable to support terminal cardiogenesis in precardiac mesoderm; however, combined BMP and FGF is capable of inducing cardiogenesis in non-precardiac mesoderm cells (Lough et al., 1996).

This report describes the additional characterization of proteins that are secreted by cultured anterior lateral plate endoderm. We demonstrate that major components of HFR endoderm secretion include trans-

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*Correspondence to: Dr. John W. Lough, Department of Cell Biology, Neurobiology, and Anatomy, Medical College of Wisconsin, 8701 W. Watertown Plank Road, Milwaukee, WI 53226.
E-mail: jlough@mcw.edu
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thyretin (TTR) and retinol binding protein (RBP), whose role in the adult vascular system is to, respectively, transport thyroid hormone and retinol (vitamin A). Although TTR and RBP mRNAs are detected in anterior lateral endoderm in vivo by stage 7, these proteins cannot mimic the cardiogenic effect of AL endoderm on cultured precardiac mesoderm cells. Transthyretin and RBP subsequently become compartmentalized within mutually exclusive myocardial structures in vivo as the heart develops. These findings are discussed in terms of possible roles for TTR and RBP during heart development and embryogenesis.

RESULTS

Metabolic Labeling Pattern of Endoderm-Secreted Proteins

The pattern of proteins synthesized and secreted by explanted anterior lateral endoderm was determined at intervals to assess whether this pattern remained constant during the culture period. Stage 5/6 explants were provided with ^{35}S-methionone prior to medium harvest to metabolically label secreted proteins, followed by SDS/PAGE separation and fluorographic imaging. As shown in Figure 1, the pattern of radioactive proteins in endoderm-conditioned medium was qualitatively similar for up to 7 days in culture, indicating that protein harvested at daily medium changes did not appreciably change. This and other determinations indicated that the most abundantly synthesized proteins migrated at approximately 200, 25, and 17 kD. We previously identified the 200-kD band as fibronectin (unpublished results), consistent with findings that precardiac mesoderm cells migrate on a fibronectin substrate (Linask and Lash, 1988).

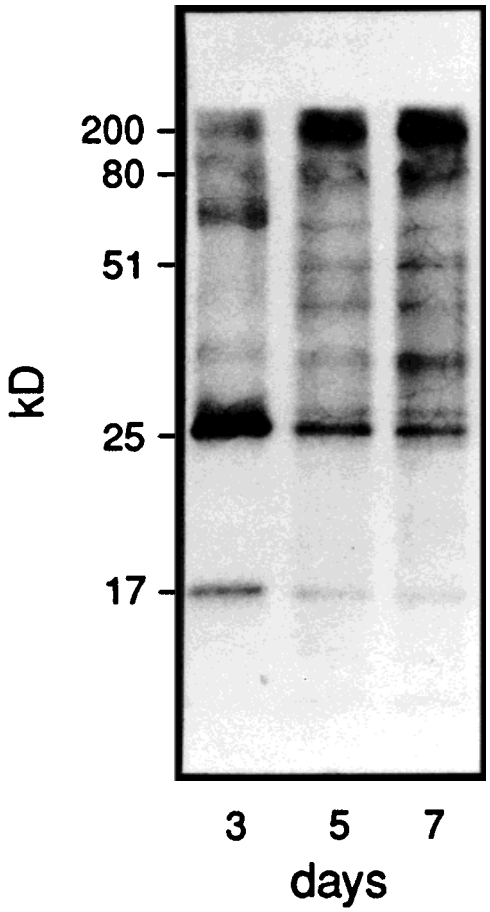


Fig. 1. Pattern of metabolically labeled proteins that are synthesized and secreted by cultured endoderm cells. Endoderm cells were explanted and cultured as described in Experimental Procedures. Eight hours before terminating cultures at days 3, 5, and 7, cells were labeled with ^{35}S-methionine. Endoderm-secreted proteins were separated on a 10% acrylamide/SDS gel, which was dried and exposed to X-ray film for six weeks. Note that the qualitative pattern of synthesized and secreted proteins was unaltered by prolonged culture, up to seven days.

Identification of the 17 kD Band as Transthyretin (TTR)

We previously used Western blotting to demonstrate that FGF-2 and activin are present in endoderm-conditioned medium (E-CM), migrating as ~17-kD proteins (Kokan-Moore et al., 1991). However, the substantial protein mass of the 17-kD band was inconsistent with the exclusive presence of these growth factors, which are present at low concentrations. It was, therefore, decided to identify the more abundant proteins in E-CM by N-terminal sequencing. As shown in Figure 2, the 17-kD band (arrowhead) was subjected to microsequencing, which revealed that its 22 N-terminal amino acids were identical to the mature N-terminus of chicken transthyretin (TTR).

Identification of the 25 kD Band as Retinol Binding Protein (RBP)

In adult serum, four identical TTR subunits circulate in a holocomplex with two molecules of retinol binding protein (RBP), a vitamin A transport protein. Because the size of the ~25-kD band(s) in Figure 1 is consistent with its identity as RBP, Western blotting was performed to assess whether a component of this band represented RBP. As shown in Figure 3 ("Western Blot"), RBP antibody recognized a ~25-kD band in endoderm-conditioned medium (E-CM lane) that co-migrated with standard human RBP (RBP lane). This result indicated high specificity of recognition by the RBP antibody, as evidenced by the ability to detect as little as 15 ng human RBP, levels that were not detectable by bulk protein staining ("Stained Gel," Fig. 3).

Expression of TTR and RBP mRNAs During Embryogenesis

To determine the onset of RBP and TTR expression, RNA from whole embryos at stages 3–21 was reverse transcribed and amplified by PCR. Complementary cDNAs representing TTR and RBP mRNAs were detected at all stages examined (Fig. 4). However, when only 1/200 as much RT product was used as template, relatively little cDNA was detected at stage 3, suggest-

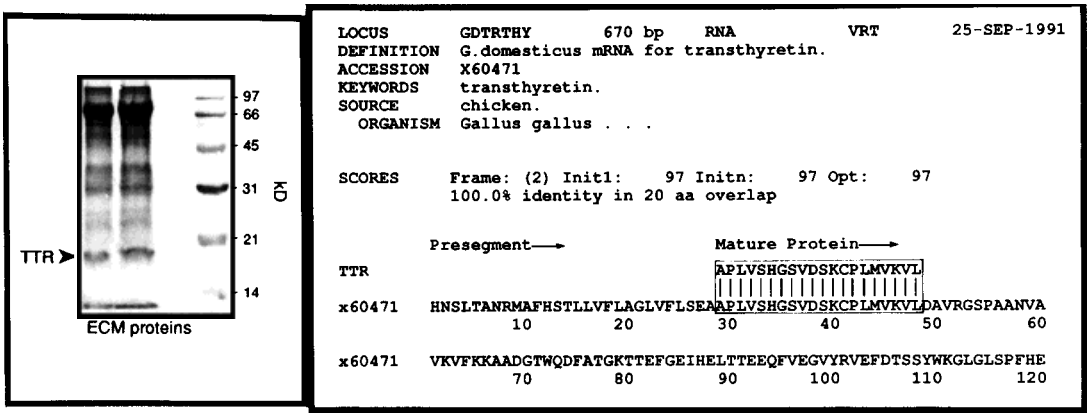


Fig. 2. N-terminal sequencing of the 17-kD band. Conditioned medium was prepared, electrophoresed, and blotted as described in Experimental Procedures. The amido black-stained 17-kD band denoted by the arrowhead was excised and subjected to N-terminal microsequencing, which revealed identity with transthyretin (TTR).

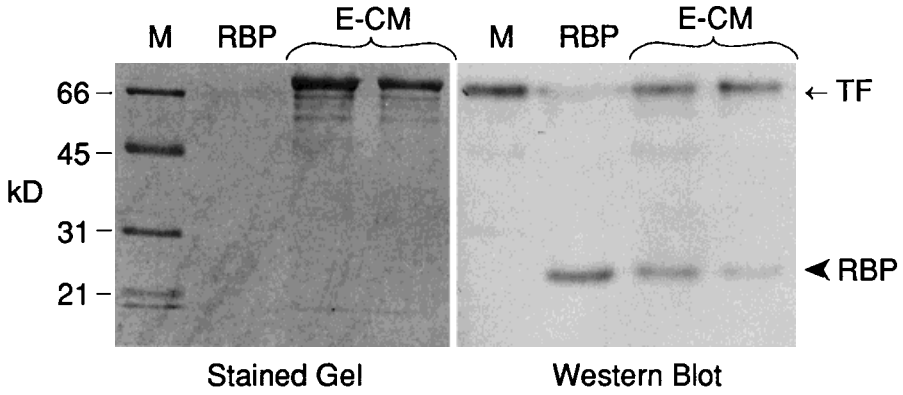


Fig. 3. Western blot analysis for RBP in endoderm-conditioned medium (E-CM). E-CM proteins were separated by 12% acrylamide/SDS-PAGE, blotted and reacted to detect RBP as described in Experimental Procedures. **Left:** Ponceau-S stained blot; the bracketed lanes contained E-CM proteins from 5.0 ml (left) and 2.5 ml (right) medium. **Right:** E-CM proteins that were recognized by RBP antiserum (1:2,000). Recognition of transferrin (TF) was presumably non-specific, caused by the abundant presence of this protein. The arrowhead denotes recognition of an E-CM protein that co-migrates with 15 ng standard RBP, which was present in the lane (RBP) adjacent to the MW markers (M).

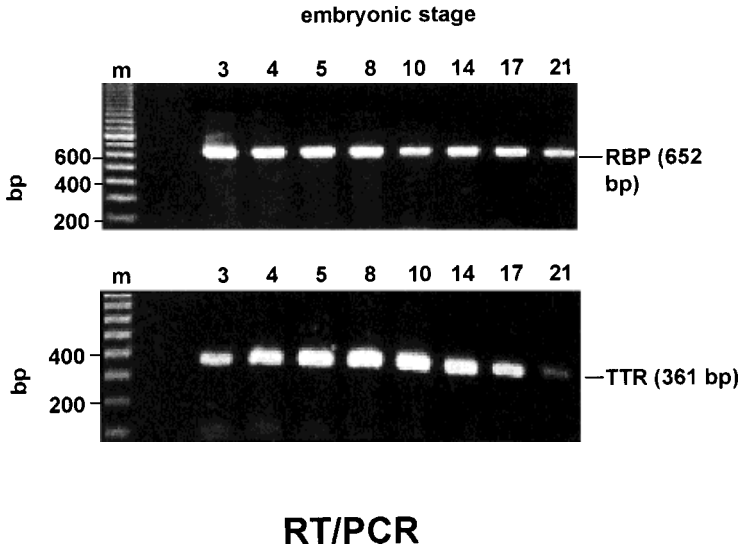


Fig. 4. RT/PCR assessment of RBP and TTR mRNA expression during embryonic development. Total RNA from embryos at the indicated stages was purified and quantitated. Exactly 1 µg of each sample was reverse transcribed and aliquots of the reverse transcription product were amplified as described in Experimental Procedures. As shown, both mRNAs were present as early as stage 3. The diminished amount of TTR at stage 21 has been a consistent finding.

ing that TTR and RBP mRNAs are up-regulated between stages 3 and 4.

In Situ Hybridization of TTR and RBP

Whole mount in situ hybridization revealed that both mRNAs were expressed in extraembryonic area at stage 6 (Fig. 5). By stage 7, these transcripts were detected in anterolateral portion of the embryo proper. Shortly thereafter (stages 8–9), these mRNAs were detected near the midline in the anterior half of the embryo, in the vicinity of the fusing myocardial tubes. At all stages, TTR and RBP mRNAs were co-localized. To identify cells that express these transcripts, hybridized embryos were sectioned. As shown in Figure 6A, B, TTR expression was confined to endoderm cells (en) between stages 7+ and 9, progressing from lateral to medial as development proceeded. Later, TTR mRNA was detected in myocardial, and perhaps endocardial, cells of the developing heart at stage 14, as shown at low magnification in Figure 6C (arrowheads) and at progressively higher magnification in Figure 7A and B. In all instances, the expression pattern for RBP mRNA was identical (not shown).

Expression of RBP Protein During Embryogenesis

To complement the assessments of RNA expression in Figures 4–7, the developmental expression of RBP protein relative to other embryonic proteins was assessed by Western blotting. Among total embryonic proteins, RBP was barely detectable at stage 7, after which increases were noted at each successive stage until stage 25 (Fig. 8, top). The temporal pattern of RBP expression in the developing heart was different: relative to heart proteins, RBP accumulation peaked at stage 14, then declined (Fig. 8, bottom).

Immunohistochemical Localization of RBP and TTR

In the adult vascular system, RBP and TTR proteins are co-localized in a holocomplex. Although Figure 5 demonstrated that TTR and RBP mRNAs are co-localized in the early embryo, it was necessary to determine whether TTR and RBP proteins were similarly co-localized, which would suggest the existence of a holocomplex at prevascular stages of development. Sections from stage 6–14 embryos were, therefore, immunostained for RBP and TTR. RBP expression is shown in Figure 9. Because RBP was only detected in the extracellular matrix, these photomicrographs were intentionally overexposed to heighten signal perception in that region (Fig. 9a,c,e). Since this practice caused cells to fluoresce above background, results using a control antibody on alternate sections are provided for comparison in each instance (Fig. 9b,d,f). RBP protein was not detected until stage 7, when it subtly appeared in the extracellular matrix between lateral plate endoderm and mesoderm cells (not shown). Upon fusion of the myocardial tubes at stage 9, RBP was observed in the "cardiac jelly" (Fig. 9a, asterisk) in addition to the extracellular matrix between endoderm and mesoderm cells (arrowhead). At stages 11 and 14, RBP staining was intensified in all extracellular matrices, especially in the cardiac jelly (Fig. 9c,e).

By contrast, TTR protein was detected slightly earlier than RBP, at stage 6 as subtle intracellular deposits in anterior lateral endoderm cells of the embryo proper (not shown); the inability to detect TTR mRNA in this region at stage 6 is presumably due to the relative insensitivity of in situ hybridization. By stage 8, TTR was observed within endoderm cells, both laterally and, in particular, within the dorsally invaginating endoderm, which lies adjacent to splanchnic mesoderm cells that soon thereafter become incorporated into the definitive myocardium (Fig. 10A). At later stages (11, 14) TTR was detected in myocardial cells of the definitive heart (Fig. 10B,C). Notably, TTR was not detected in ventral foregut endoderm at these stages (not shown).

Do RBP and TTR Affect In Vitro Cardiogenesis?

Because these expression patterns suggested a possible role in heart development, it was determined whether RBP and/or TTR could replace the ability of HFR endoderm, or of growth factors that mimic its activity, to support the terminal differentiation of precardiac mesoderm. This approach was based on the rationale that although RBP and TTR are not growth factors, they may function as growth factors by transporting retinol and thyroid hormone. In contrast to the cardiogenic effect of HFR endoderm (not shown) or of FGF, neither individually applied nor combined RBP/TTR was able to support terminal cardiogenesis in explanted precardiac mesoderm (Fig. 11). Moreover,

Fig. 5. Whole-mount in situ hybridization of TTR and RBP mRNAs. In situ hybridization was performed as described in Experimental Procedures using digoxigenin-labeled antisense riboprobes. Note that the expression of both mRNAs is identical, and that intraembryonic expression beginning at stage 8 essentially comprises the anterior half of the embryo, progressing from medial to lateral as development ensues. Control hybridizations using a sense riboprobe exhibited no signal (not shown).

Fig. 6. Expression of TTR mRNA in transverse-sections from whole-mount in situ hybridized embryos. Whole-mount in situ hybridized embryos were transversely sectioned through the heart region as described in Experimental Procedures. Note that TTR mRNA was restricted to endoderm cells (en) between stages 7+ (**A**) and 9 (**B**), progressing toward the midline as development ensued. **C:** At stage 14, while endoderm (en) exhibited the most intense signal, mRNA was also detected in cells of the myocardial and endothelial layers as indicated by the arrowheads and shown at higher magnification in Fig. 7. The pattern of RBP mRNA expression was identical at each stage. nt = neural tube; spm = splanchnic mesoderm. Bars = 100 μm.

Fig. 7. High-magnification resolution of TTR mRNA in transverse-sections from whole-mount in situ hybridized embryos at stage 14. **A** and **B** are higher magnification images of the area indicated by the arrowheads in Figure 6C to illustrate the expression of TTR mRNA by myocardial (m) and endothelial (e) cells of the developing heart. Bars = 200 μm (A), 400 μm (B). en = endoderm; f = foregut.

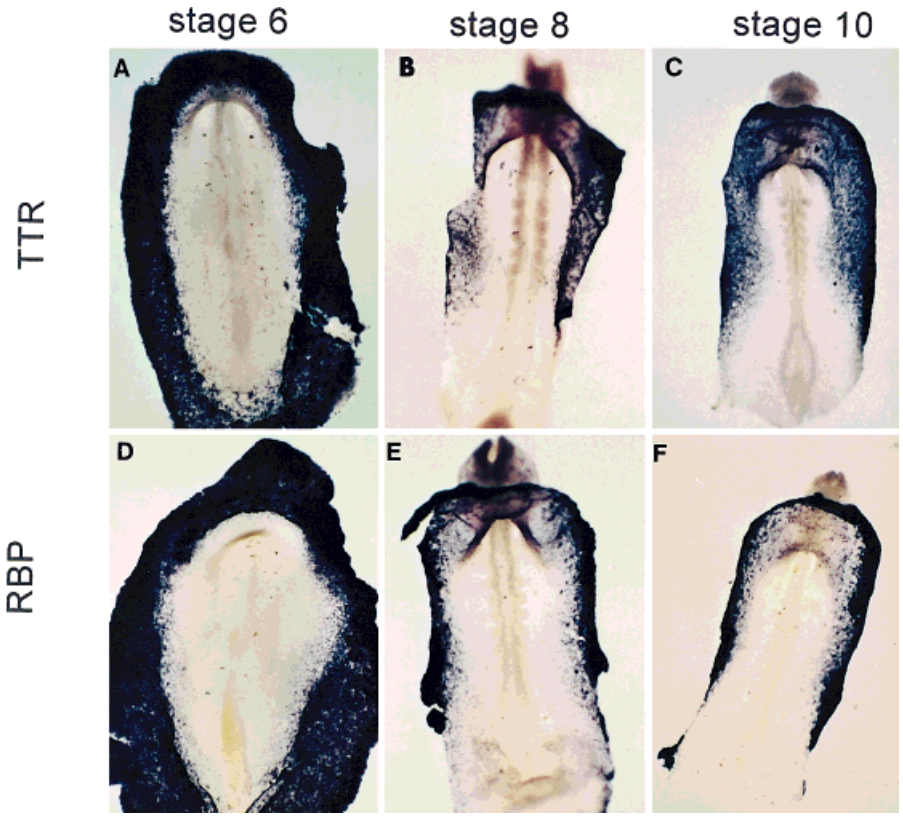
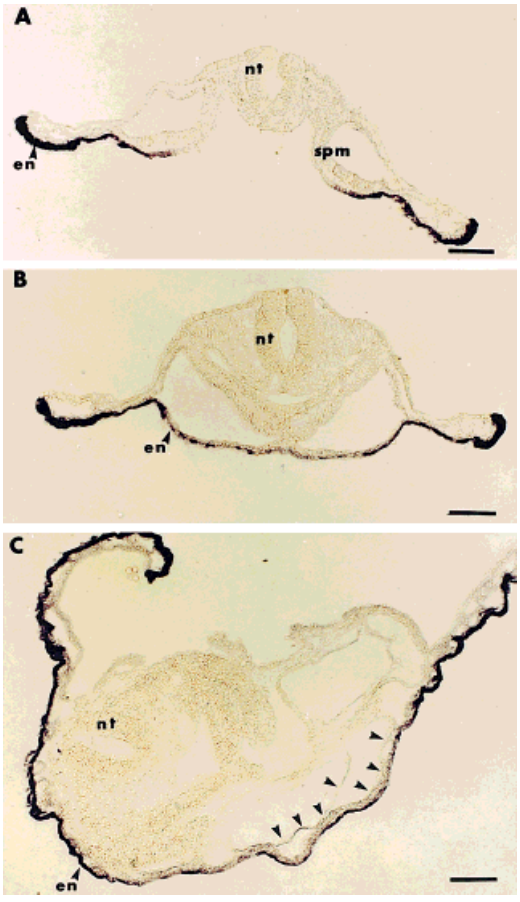


Figure 5.



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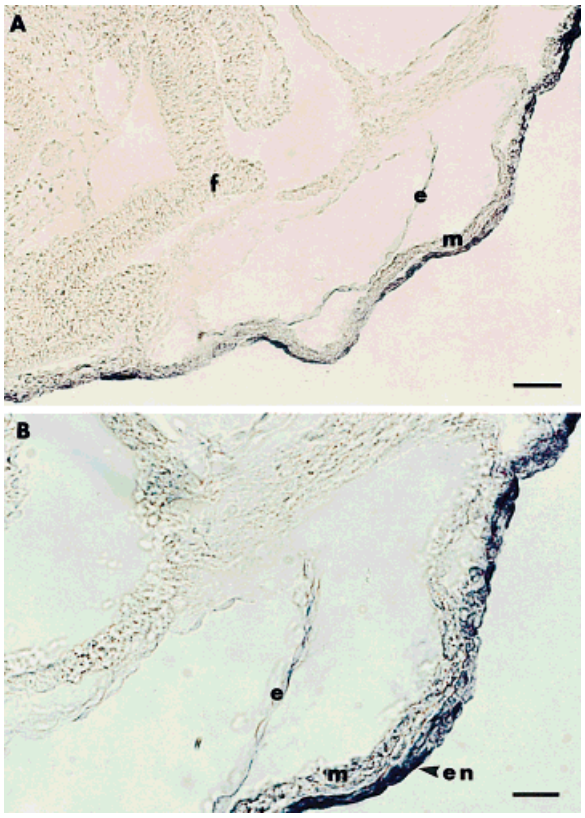

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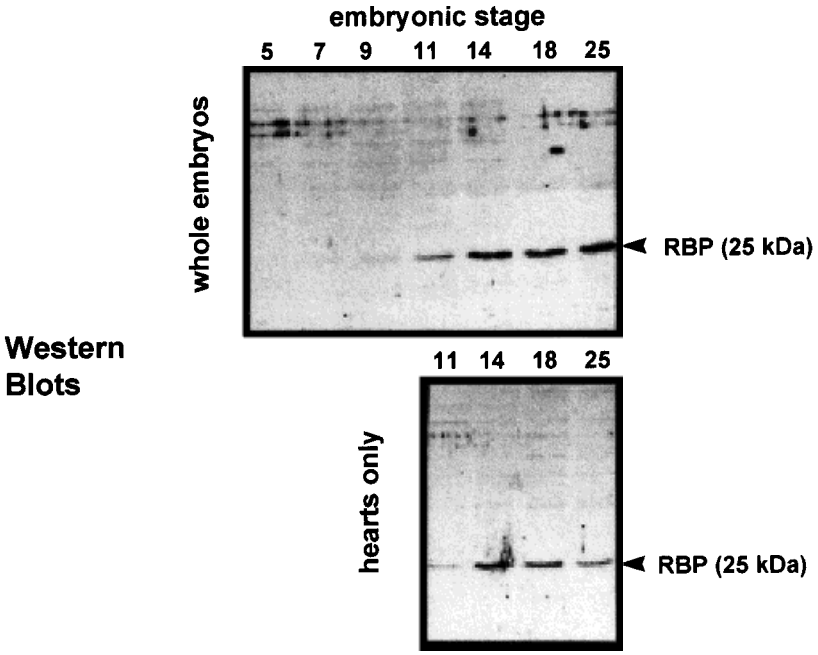

Fig. 8. Western blot analyses of RBP differentiation in samples from whole embryos and isolated hearts. Exactly 50 μg protein from whole embryos (**top**) and isolated hearts (**bottom**) at the indicated stages was separated and immunoreacted for RBP as described in Experimental Procedures.

various combinations of RBP and TTR with retinol and/or thyroid hormone (T3) had no effect (not shown).

DISCUSSION

Recent findings have supported the notion that endoderm cells in the anterior lateral plate of the stage 5/6 avian embryo potently and specifically support the terminal differentiation of precardiac mesoderm (Sugi and Lough, 1994; Schultheiss et al., 1995). The identification of endoderm products that mediate this process remains a compelling task. We previously reported that members of the FGF family as well as activin-A are manufactured by anterior lateral endoderm cells (Kokan-Moore et al., 1991; Parlow et al., 1991). The findings presented here indicate that transthyretin (TTR) and retinol binding protein (RBP), which reside in a holocomplex to, respectively, transport thyroid hormone and vitamin A in the adult circulation, are major products of embryonic endoderm.

Transthyretin and retinol binding protein appear in the embryo prior to the onset of vascular system development. Using immunohistochemistry, TTR protein was detected in embryonic endoderm at stage 6 and RBP was detected in the adjacent extracellular matrix at stage 7. Previous in situ hybridization studies in rat embryos detected TTR mRNA in visceral endoderm by day e7 (Murakami et al., 1987; Makover et al., 1989) and later in the choroid plexus and brain (e10/11; Thomas et al., 1988; Makover et al., 1989; Cavallaro et al., 1993) and visceral yolk sac endoderm (e14; Soprano et al., 1986). During chicken oogenesis, retinol is transported to the yolk from the adult circulation (Vieira and Schneider, 1993; Vieira et al., 1995b) by RBP, which is not expressed by oocytes or other ovarian cells (Vieira et al., 1995a). Our results extend these findings, indicating that extraembryonic expression of RBP and TTR, which apparently begins between fertilization and the onset of gastrulation, is manifest by in situ hybridization at stage 6 in endoderm overlying the yolk (Fig. 5). Shortly thereafter, these mRNAs are detected in the embryo per se, sequentially appearing in endoderm, heart, and liver cells. These findings suggest a requirement for the localized availability of transport proteins during oogenesis and early embryonic development.

Vitamin A and thyroid hormone are critical for normal embryogenesis. Retinoic acid (RA), the major bioactive metabolic product of vitamin A, regulates the differentiation and morphogenesis of various embryonic tissues (reviewed in Morriss-Kay and Sokolova, 1996; Hoffmann and Eichele, 1994), including the heart (reviewed in Smith and Dickman, 1997). Thyroid hormone, in addition to regulating metabolic processes, affects neuron and cardiac myocyte differentiation in the developing embryo. Both hormones regulate target gene transcription by binding to cognate nuclear steroid receptors that share common heterodimeric partners, enabling cross-talk via related cis-acting response elements in the promoters of target genes (reviewed in Mangelsdorf et al., 1994). This interrelated function suggests that the circulatory holocomplex transport system for these pro-hormones evolved to ensure coordinated transcriptional activity in the adult organism. In the early embryo, the presence of TTR and RBP in the endoderm and yolk sac prior to establishment of the circulatory system may enable the acceptance of ligand directly from the yolk, which is the sole source of retinol and the predominant source of thyroid hormone (Prati et al., 1992). From endoderm, RBP and TTR could distribute their respective ligands directly to overlying embryonic structures, such as the developing heart.

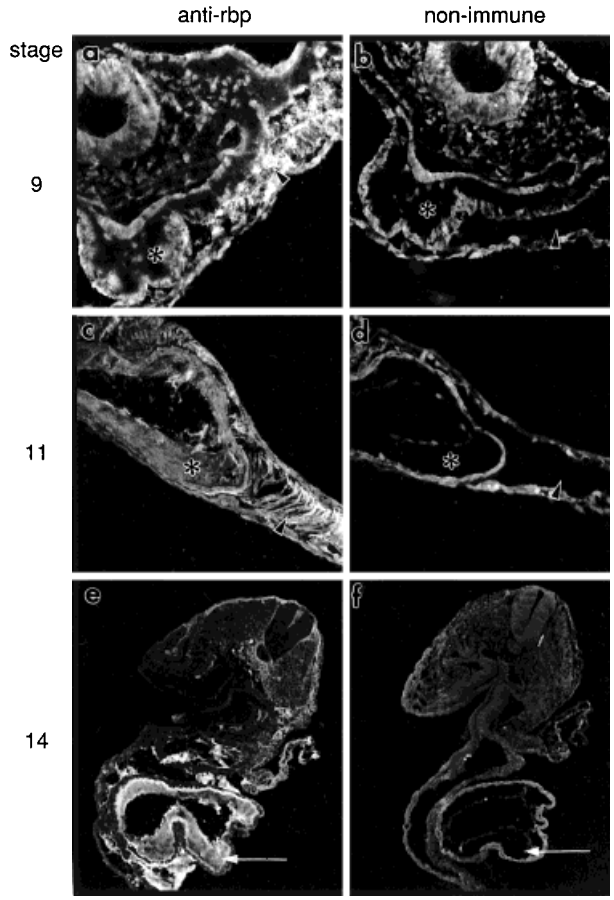

Fig. 9. Immunohistochemical localization of RBP during embryonic development. Embryos at the indicated stages were cryopreserved, sectioned, and immunostained using the same antiserum as in the Western blotting assessment (Fig. 8); details are described in Experimental Procedures. Because all specific immunostaining was detected in the extracellular matrix, photomicrographs were deliberately overexposed to heighten signal from the matrix. asterisks and arrows = cardiac jelly; arrowheads = extracellular matrix.

Despite our finding that neither RBP nor TTR are *sufficient* to support cardiogenesis in vitro, it is possible that their presence in endoderm is *necessary* to support cardiogenic processes, in addition to providing broad nutritional and transport functions in the embryo.

In adults, an advantage of the circulatory RBP/TTR holocomplex is that monomer loss in the glomerular kidney filtrate is minimized (reviewed in Soprano and Blaner, 1994). The absence of this prospect in the early embryo may enable RBP and TTR to be separately localized in order to exert their specific functions. It is accordingly speculated that the localization of enriched deposits of RBP in cardiac jelly and TTR in myocardial cells reflects different functions for their ligands during heart development. For example, thyroid hormones modulate the expression of cardiac myofibril components such as α-myosin heavy chain (Izumo and Mahdavi, 1988), suggesting that intracellular TTR may regulate thyroid hormone availability in the myocardium; other possibilities would include a role for TTR in vasculogenesis. Overwhelming evidence indicates that precisely regulated levels of vitamin-A and RA are required for normal cardiovascular development. In avians, elevated RA causes myocardial defects including disruption of the cardiogenic crescent, abnormal heart tube looping, and cardia bifida (Osmond et al., 1991; Chen et al., 1992), as well as expansion of atrial-specific gene expression into ventricular-fated cells (Yutzey et al., 1995). In older hearts, excessive RA disrupts formation of the outflow tract septum by inhibiting epithelial-mesenchymal transformation of the endocardium (Nakajima et al., 1996). Vitamin A deficiency is lethal to avian embryos (Dersch and Zile, 1993; Twal et al., 1995), resulting in absence of RA and nuclear retinoic acid receptor in the HFR (Dong and Zile, 1995) and failure to link extraembryonic vasculature with the inflow tract (Heine et al., 1985; Dersch and Zile, 1993). In mice, null mutation of retinoic acid receptor genes or RA deficiency causes embryonic lethality due to cardiac failure (Sucov et al., 1994; Gruber et al., 1996; Kastner et al., 1997a,b). These mutations are accompanied by reduced cardiac myocyte proliferation and increased myofibril formation that is suggestive of precocious cardiac myocyte differentiation (Kastner et al., 1997a), as well as loss of NADH ubiquinone reductase activity and impaired oxidative phosphorylation (Ruiz-Lozano et al., 1998). Acute sensitivity of the developing heart to RA is perhaps most dramatically illustrated in *Xenopus*, wherein modest doses delete cardiac structures (Drysdale et al., 1997) and in zebrafish, where RA disrupts arterial-venous patterning (Stanier and Fishman, 1992). From these findings it is speculated that the observed localization of RBP in the cardiac jelly creates a repository for the regulated delivery of retinoid delivery to developing cardiac tissue; an important role for RBP in this regard is suggested by observations that antisense-mediated depletion of RBP suppresses RA-dependent activity of RAR promoter in the developing mouse heart (Båvik et al., 1996). Subsequent transport of RBP across the endocardial monolayer would deliver this protein to the embryonic circulation.

Elucidation of the mechanistic roles of RBP and TTR during early development is difficult to approach using the avian model. Clarification of these proteins' function(s) will require their tissue- and stage-specific deletion from the in vivo embryo. Although a TTR null mutant mouse has been made (Wei et al., 1995), these embryos appear essentially normal, most likely due to functional redundancy provided by multiple thyroid hormone transport proteins. A RBP mutant mouse has not been constructed. Availability of the latter, or experiments using retroviral vectors to mis-express RBP in selected regions of the in vivo avian embryo, will be of paramount value toward elucidating how this protein affects early embryogenesis and cardiogenesis.

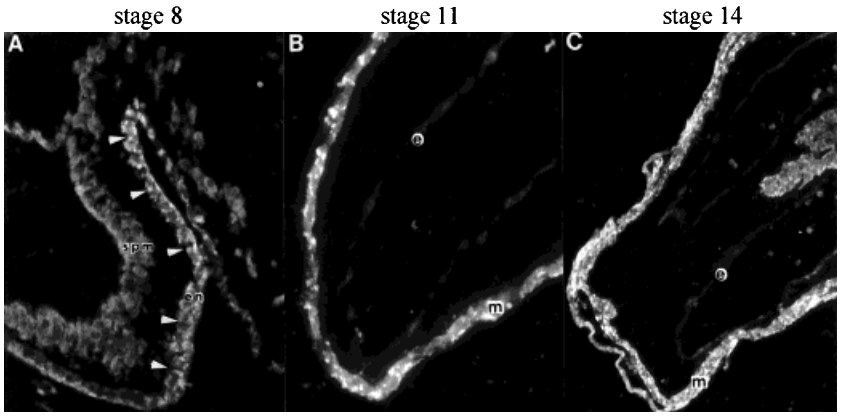

Fig. 10. Immunohistochemical localization of TTR during embryonic development. Embryos at the indicated stages were prepared as described for Figure 9 and immunostained using TTR antiserum. The section in **A** begins at the midline of the embryo; arrowheads depict TTR in invaginated anterior lateral endoderm (en), which is adjacent to myocardial precursor cells in the splanchnic mesoderm (spm). **B** and **C** show sections through the definitive heart: e = endocardium; m = myocardium.

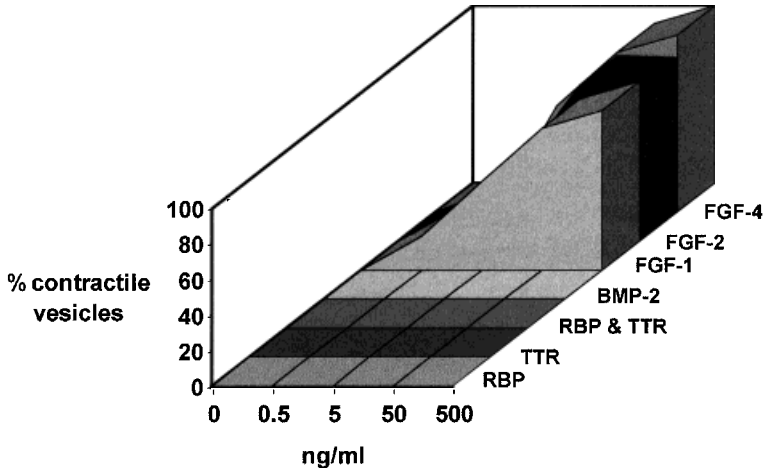

Fig. 11. RBP and TTR cannot induce in vitro cardiogenesis. Precardiac mesoderm, alone, was explanted from stage 6 embryos as described in Experimental Procedures and cultured in defined medium supplemented with the indicated protein supplements. The percentage of explants that differentiated into contractile vesicles at the indicated dosage of each purified growth factor was the average of at least six independent assessments.

EXPERIMENTAL PROCEDURES

Explant Culture of Anterior Lateral Plate Endoderm/Precardiac Mesoderm

Details of this procedure are described in previous publications (Sugi and Lough, 1994, 1995).

Harvest of Endoderm-Conditioned Medium (E-CM)

To obtain sufficient material for characterization, medium was harvested from cultures at daily intervals up to 7 days, since one-dimensional fluorographic SDS/PAGE analysis of ^{35}S-methionine-labeled proteins had shown that the pattern of secreted E-CM proteins is independent of duration in culture. Approximately 100 ml were harvested over a 10-week period and stored at −80°C in the presence of a protease inhibitor cocktail consisting of 0.5 μg/ml pepstatin, 1.0 μg/ml leupeptin, 0.1 mM phenylmethylsulfonylfluoride, and 100 U/ml aprotinin.

Microsequencing

Aliquots of 5 ml E-CM were dialyzed against ice-cold 2% acetic acid and concentrated to dryness, followed by determination of total protein. Ten micrograms of protein were separated on a 12% acrylamide/SDS gel, electrophoretically transferred to Immobilon-P PVDF (0.45 μm pore size; Millipore, Bedford, MA, cat. no. IPVH 091 20) according to the method of Matsudaira (1987) and stained with Amido Black. Microsequencing of the excised 17-kD protein band was performed by N-terminal Edman degradation. The sequence was compared with the GenBank/EMBO protein database.

Western Blotting

Aliquots of E-CM were dialyzed and concentrated to dryness as described previously and samples were separated on a 12% acrylamide:bis (30:0.8)/SDS gel. For a positive control, 15 ng pure human retinol-binding protein (RBP; Calbiochem, La Jolla, CA, cat. no. 554702) was utilized. Separated proteins were electroblotted onto nitrocellulose (Schleicher & Schuell, Keene, NH, BA85), blocked and reacted with the primary antiserum, sheep anti-RBP (1:2,000 in TBS-T; Chemicon, Temecula, CA, cat. no. AB734) for 4 hr, then with dichlorotriazinyl (DTAF)-conjugated rabbit anti-sheep antibody (1:2,000 in TBS-T) for 1 hr at room temperature. Finally, the blot was incubated with protein A-horseradish peroxidase conjugate (1:4,000; Bio-Rad, Richmond, CA, cat. no. 170-6522) for 30 min at room temperature and covered with a 1:1 mixture of

ECL detection reagent 1 (Amersham, Arlington Heights, IL, cat. no. RPN 2106) and ECL detection reagent 2 for 1 min at room temperature. Antigens were localized by exposing the membrane to Hyperfilm-ECL for intervals up to 5 min.

Immunohistochemistry

Importantly, embryos were fixed by cryopreservation (Kitten et al., 1987) to preserve the extracellular matrix; details of this procedure are described in a previous communication (Sugi and Lough, 1992). Paraplast-embedded embryos were sectioned at 5 μm and mounted to subbed slides; de-paraffinized sections were incubated at 4°C with 5% BSA/PBS overnight to block non-specific binding.

Retinol binding protein immunostaining. All dilutions and washes were performed using the blocking solution (5% BSA in PBS). The primary antiserum was sheep anti-RBP (1:500; Chemicon Int., no. AB734) or normal sheep serum (1:500; Sigma, St. Louis, MO, no. 5-7773), applied in a humid chamber at 4°C overnight. The secondary antibody, Texas red-conjugated rabbit anti-sheep (1:50), was applied for 1 hr at room temperature. After a final blocking step using whole goat serum (1:50; Cappel, Malvern, PA, no. 55984) for 30 min at RT, FITC-conjugated affinity-purified goat anti-rabbit antiserum (1:50; Cappel no. 55662) was applied for 1 hr at room temperature.

Transthyretin immunostaining. The primary antiserum was rabbit anti-TTR (1:500) prepared by Dr. W.S. Blaner (Columbia University, NY) or normal rabbit serum (1:500; Cappel, no. 5012-1380), applied in a humid chamber at 4°C overnight. The secondary antibody, FITC-conjugated goat anti-rabbit IgG (1:100; Cappel, no. 55662) was applied at RT for 1 hr.

In Situ Hybridization

TTR riboprobe. Complementary DNA for chicken transthyretin was obtained by RT/PCR. For the PCR step, forward and reverse primers (1 μM each), respectively, corresponded to nucleotides 72–91 and 433–452 chicken TTR (Duan et al., 1991). The forward primer was 5′-TTCTCTCCGAAGCTGCACCA-3′; the reverse primer was 5′-AGGACTGAGGAGAGCAGCGA-3′. The PCR reaction was performed using 40 cycles of denaturation (1 min, 94°C) annealing (2 min, 55°C) and extension (3 min, 72°C). The 380-bp PCR product was gel-purified and cloned into pCRScript (Stratagene, La Jolla, CA, no. 211190). Clones containing cDNA inserts in both orientations were obtained, as verified by sequencing. Because T7 RNA polymerase (Boehringer-Mannheim, Indianapolis, IN, no. 881-767) was determined to most efficiently incorporate the digoxigenin-11-UTP nucleotide analog (Boehringer-Mannheim, no. 1209-256) into riboprobe transcripts, antisense and sense riboprobes containing digoxigenin were both synthesized by activating the T7 promoter in clones containing appropriately oriented inserts.

RBP riboprobe. Complementary DNA for chicken RBP was similarly obtained. For the PCR step, forward and reverse primers respectively corresponding to nucleotides 16–35 and 668–687 chicken RBP (Vieira et al., 1995a) were used at a concentration of 1 μM each. The forward primer was 5′-CGGAGCTGTCTGGACAGGAT-3′; the reverse primer was 5′-CCATGGTACTGTTACACGGT-3′. All other procedures were performed as described for TTR.

Hybridization. Whole mount embryo in situ hybridization was performed as described by Harland (1991) and modified by Knecht et al. (1995), which should be consulted for details. Embryos were fixed in fresh 4% paraformaldehyde overnight, followed by rinsing with PBS and series dehydration, culminating in 100% MeOH. If necessary, embryos were stored at −20°C. Prior to hybridization, embryos were rehydrated in PBS/0.1% Tween-20, which was used for all subsequent washes, and digested with 1.5 μg/ml proteinase-K for exactly 5 min at room temperature, followed by washing and treatment with 0.1 M triethanolamine containing 12.5 μl acetic anhydride/5 ml. Embryos were post-fixed in 4% fresh paraformaldehyde, washed and transferred to siliconized Eppendorf tubes containing hybridization buffer (20 mM Tris [pH 7.4], 50% formamide, 0.33 M NaCl, 5 mM EDTA, 10% dextran sulfate, 1× Denhardt's, 0.5 mg/ml yeast tRNA in DEPC-treated DW). Hybridization was performed overnight at 50°C using 1 μg/ml antisense or sense (control) riboprobe. After hybridization, embryos were washed and in 50% formamide/2× SSC/0.1% SDS for 20 min at 65°C followed by 10 mM Tris (pH 7.5)/400 mM NaCl/5 mM EDTA (2×/10 min/37°C). Embryos were then digested with RNase-A (20 μg/ml), washed and blocked with 10% normal goat serum, followed by addition of anti-DIG antibody (1:500; Boehringer-Mannheim #1175-041). After overnight incubation at 4°C the embryos were washed and reacted with NBT/X-phosphate according to the manufacturer's recommendations (Boehringer-Mannheim #1175-041). Color was developed for 2 hr and the reaction was terminated by placing the embryos in fresh 4% paraformaldehyde (20 min/RT), followed by transfer to 1× PBS for photography and subsequent storage (4°C). Following color development, cross-sectional analysis was performed by embedding embryos in Paraplast, sectioning (20 μm), clearing with Americlear and observing with bright-field microscopy.

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REFERENCES

Båvik C, Ward SJ, Chambon P. Developmental abnormalities in cultured mouse embryos deprived of retinoic acid by inhibition of

yolk-sac retinol binding protein synthesis. Proc Natl Acad Sci USA 1996;93:3110–3114.
Cavallaro T, Martone RL, Stylianopoulou F, Herbert J. Differential expression of the insulin-like growth factor II and transthyretin genes in the developing rat choroid plexus. J Neuropathol Exp Neurol 1993;52:153–162.
Chen Y, Huang L, Russo F, Solursh M. Retinoic acid is enriched in Hensen's node and is developmentally regulated in the early chicken embryo. Proc Natl Acad Sci USA 1992;89:10056–10059.
Dersch H, Zile MH. Induction of normal cardiovascular development in the vitamin A-deprived quail embryo by natural retinoids. Dev Biol 1993;160:424–433.
Dong D, Zile MH. Endogenous retinoids in the early avian embryo. Biochem Biophys Res Commun 1995;217:1026–1031.
Drysdale TA, Patterson KD, Saha M, Krieg PA. Retinoic acid can block differentiation of the myocardium after heart specification. Dev Biol 1997;188:205–215.
Duan W, Achen MG, Richardson SJ, Lawrence MC, Wettenhall REH, Jaworowski A, Schreiber G. Isolation, characterization, cDNA cloning and gene expression of an avian transthyretin. Eur J Biochem 1991;200:679–687.
Gruber PJ, Kubalak SW, Pexieder T, Sucov HM, Evans RM, Chien KR. RXRα deficiency confers genetic susceptibility for aortic sac, conotruncal, atrioventricular cushion, and ventricular muscle defects in mice. J Clin Invest 1996;98:1332–1343.
Harland RM. In situ hybridization: An improved whole mount method for Xenopus embryos. Methods Cell Biol 1991;36:685–695.
Heine UI, Roberts AB, Munoz EF, Roche NS, Sporn MB. Effects of retinoid deficiency on the development of the heart and vascular system of the quail embryo. Virchows Arch 1985;50:135–152.
Hoffmann C, Eichele G. Retinoids in development. In: Sporn MB, Roberts AB, Goodman DS, eds. The Retinoids: Biology, Chemistry and Medicine, 2nd ed. New York: Raven Press, 1994;387–441.
Izumo S, Mahdavi V. Thyroid hormone receptor α isoforms generated by alternative splicing differentially activate myosin HC gene transcription. Nature 1988;334:593–595.
Kastner P, Messaddeq N, Mark M, Wendling O, Grondona JM, Ward S, Ghyselinck N, Chambon P. Vitamin A deficiency and mutations of RXRα, RXRβ and RARα lead to early differentiation of embryonic ventricular cardiomyocytes. Development 1997a;124:4749–4758.
Kastner P, Mark M, Ghyselinck N, Krezel W, Dupe V, Grondona JM, Chambon P. Genetic evidence that the retinoid signal is transduced by heterodimeric RXR/RAR functional units during mouse development. Development 1997b;124:313–326.
Kitten GT, Markwald RR, Bolender DL. Distribution of basement membrane antigens in cryopreserved early embryonic hearts. Anat Rec 1987;217:379–390.
Knecht AK, Good PJ, Dawid IB, Harland RM. Dorsal-ventral patterning and differentiation of noggin-induced neural tissue in the absence of mesoderm. Development 1995;121:1927–1936.
Kokan-Moore NP, Bolender DL, Lough J. Secretion of inhibin β_A by endoderm cultured from early embryonic chicken. Dev Biol 1991;146: 242–245.
Linask KK, Lash JW. A role for fibronectin in the migration of avian precardiac cells. I. Dose-dependent effects of fibronectin antibody. Dev Biol 1988;129:315–323.
Lough J, Barron M, Brogley M, Sugi Y, Bolender DL, Zhu X. Combined BMP-2 and FGF-4, but neither factor alone, induces cardiogenesis in non-precardiac embryonic mesoderm. Dev Biol 1996;178:198–202.
Makover A, Soprano DR, Wyatt ML, Goodman DS. An in situ-hybridization study of the localization of retinol-binding protein and transthyretin messenger RNAs during fetal development in the rat. Differentiation 1989;40:17–25.
Mangelsdorf DJ, Umesono K, Evans RM. The retinoid receptors. In: Sporn MB, Roberts AB, Goodman DS, eds. The Retinoids: Biology, Chemistry and Medicine, 2nd ed. New York: Raven Press, Ltd, 1994;319–349.
Matsudaira P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J Biol Chem 1987;262:10035–10038.
Morriss-Kay GM, Sokolova N. Embryonic development and pattern formation. FASEB J 1996;10:961–968.
Murakami T, Yasuda Y, Mita S, Maeda S, Shimada K, Fujimoto T, Araki S. Prealbumin gene expression during mouse development studied by in situ hybridization. Cell Differ. 1987;22:1–10.
Nakajima Y, Morishima M, Nakazawa M, Momma K. Inhibition of outflow cushion mesenchyme formation in retinoic acid-induced complete transposition of the great arteries. Cardiovasc Res 1996;31: E77–E85.
Osmond M, Butler A, Voon F, Bellairs R. The effects of retinoic acid on heart formation in the early chick embryo. Development 1991;113: 1405–1417.
Parlow M, Bolender D, Kokan-Moore N, Lough J. Localization of bFGF-like proteins as punctate inclusions in the preseptation myocardium of the chicken embryo. Dev Biol 1991;146:139–147.
Prati M, Calvo R, Morreale G, de Escobar EG. L-Thyroxine and 3,5,3′-triiodothyronine concentrations in the chicken egg and in the embryo before and after the onset of thyroid function. Endocrinology 1992;130:2651–2659.
Ruiz-Lozano P, Smith SM, Perkins G, Kubalak SW, Boss GR, Sucov HM, Evans RM, Chien KR. Energy deprivation and a deficiency in downstream metabolic target genes during the onset of embryonic heart failure in RXRα−/− embryos. Development 1998;125:533–544.
Schultheiss TM, Xydas S, Lassar AB. Induction of avian cardiac myogenesis by anterior endoderm. Development 1995;121:4203–4214.
Schultheiss TM, Burch JBE, Lassar AB. A role for bone morphogenetic proteins in the induction of cardiac myogenesis. Genes Dev 1997;11: 451–462.
Smith SM, Dickman ED. New insights into retinoid signaling in cardiac development and physiology. Trends Cardiovasc Med 1997;7:53–58.
Soprano DR, Soprano KJ, Goodman DW. Retinol-binding protein and transthyretin mRNA levels in visceral yolk sac and liver during fetal development in the rat. Proc Natl Acad Sci USA 1986;83:7330–7334.
Soprano DR, Blaner WS. Plasma retinol-binding protein. In: Sporn MB, Roberts AB, Goodman DS, eds. The Retinoids: Biology, Chemistry and Medicine, 2nd edition. New York: Raven Press, Ltd, 1994;257–281.
Stainier DYR, Fishman MC. Patterning the zebrafish heart tube: acquisition of anteroposterior polarity. Dev Biol 1992;153:91–101.
Sucov H, Dyson E, Gumeringer C, Price J, Chien K, Evans R. RXR mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. Genes Dev 1994;8:1007–1018.
Sugi Y, Lough J. Onset of expression and regional deposition of alpha-smooth and sarcomeric actin during avian heart development. Dev Dyn 1992;193:116–124.
Sugi Y, Lough J. Anterior endoderm is a specific effector of terminal cardiac myocyte differentiation of cells from the embryonic heart forming region. Dev Dyn 1994;200:155–162.
Sugi Y, Lough J. Activin-A and FGF-2 mimic the inductive effects of anterior endoderm on terminal cardiac myogenesis in vitro. Dev Biol 1995;169:567–574.
Thomas T, Power B, Hudson P, Schreiber G, Dziadek M. The expression of transthyretin mRNA in the developing rat brain. Dev Biol 1988;128:415–427.
Twal W, Roze L, Zile MH. Anti-retinoic acid monoclonal antibody localizes all-trans-retinoic acid in target cells and blocks normal development in early quail embryo. Dev Biol 1995;225–234.
Vieira AV, Schneider WJ. Transport and uptake of retinol during chicken oocyte growth. Biochim Biophys Acta 1993;1169:250–256.
Vieira AV, Kuchler K, Schneider WJ. Retinol in avian oogenesis: Molecular properties of the carrier protein. DNA Cell Biol 1995a;14: 403–410.
Vieira AV, Sanders EJ, Schneider WJ. Transport of serum transthyretin into chicken oocytes. A receptor-mediated mechanism. J Biol Chem 1995b;270:2952–2956.
Wei S, Episkopou V, Piantedosi R, Maeda S, Shimada K, Gottesman ME, Blaner WS. Studies on the metabolism of retinol and retinol-binding protein in transthyretin-deficient mice produced by homologous recombination. J Biol Chem 1995;270:866–870.
Yutzey KE, Gannon M, Bader D. Diversification of cardiomyogenic cell lineages in vitro. Dev Biol 1995;170:531–541.
Zhu X, Sasse J, McAllister D, Lough J. Evidence that fibroblast growth factors 1 and 4 participate in regulation of cardiogenesis. Dev Dyn 1996;207:429–438.