DEVELOPMENTAL BIOLOGY **173,** 39–50 (1996)
Article No. 0005

Left–Right Asymmetric Localization of Flectin in the Extracellular Matrix during Heart Looping

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The early embryo is initially bilaterally symmetrical. One of the first distinct indications of asymmetry in the embryo occurs during heart looping. The midline tubular heart begins to bend to the right to form a C-shaped structure around 30 hr of development in the avian model. A molecular basis for heart asymmetry and direction of looping is not known, although factors inherent to the myocardium are believed to underlie looping. A left–right asymmetric localization of a specific molecule in the bilateral heart forming regions has not been reported previously. One molecule that we are calling flectin (*flectere,* in L., to bend or to loop) shows a bilateral asymmetric localization early in the heart forming mesoderm and continues to be expressed asymmetrically in a highly organized manner in the cardiac jelly during heart looping. This large extracellular matrix molecule has been identified using a monoclonal antibody F-22 (Mieziewska *et al.,* 1994a,b). Flectin shows a discrete spatiotemporal pattern of extracellular matrix expression during avian heart development. An asymmetric expression of flectin is observed during heart development at stage 7+/8− (approximately at 24 hr of development around the 3-somite stage). It is predominantly expressed in the left precardiac mesoderm at this developmental period. Between stages 12 and 14, flectin continues to be asymmetrically expressed in the myocardium and is localized at high levels on the basal side of the myocardium and within the cardiac jelly extending to the endocardial cell surfaces. In the same plane of the looping part of the heart it is differentially organized within the cardiac jelly on the convex side and in the outer loop areas. A reduced expression is apparent anteriorly and posteriorly along the tubular heart. The initial asymmetry of localization is maintained throughout the tubular heart. At stage 22 (Embryonic Day 3.5), intensity of immunolocalization of flectin is significantly decreased, with left–right asymmetry becoming less discernible or absent. It again is expressed in Day 10 embryonic hearts. Flectin expression appears to be modulated by retinoids. In vitamin A-deficient quail embryonic hearts that do not loop (Dersch and Zile, 1993; Twal *et al.,* 1995), flectin protein expression is decreased and disorganized, as are other extracellular matrix components comprising the cardiac jelly.

INTRODUCTION

Left–right asymmetries appear last during the development of the embryonic axes and are involved in the positioning and structure of organs such as the heart and of derivatives of the gut tube such as lungs, spleen, and stomach. Asymmetry has been reported early in development in association with the primitive streak stage. The Hensen's Node is described as being asymmetric, with the righthand side being bigger than the left (cited in Hoyle *et al.*, 1992). A more noticeable indication of left–right handedness is observed during the looping of the heart (Stalsberg, 1969). Consistently, the heart curvature is to the right. From previous experimentation a left-sided dominance appears to lead to heart looping and to determine the direction of the curvature (Wilens, 1955; Castro-Quezada *et al.*, 1972; Hoyle *et al.*, 1992). Specific molecular factors that may define heart looping have not been identified, but classes of molecules have been implicated. Yost has reported that inhibition of proteoglycan synthesis (Yost, 1990) or perturbation of the extracellular matrix by microinjection of Arg-Gly-Asp peptides (Yost, 1992) results in global randomization of left–

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0012-1606/96 $12.00

right asymmetry. These results suggest that the extracellular matrix contains axial information and is involved in the determination of the direction of heart looping.

The possibility that early differences between the right and left areas of the precardiac mesoderm could affect the looping direction was analyzed by Salazar del Rio (1974). In his studies using avian embryos he constructed embryos with two right or two left areas of precardiac mesoderm at stage 5 and followed subsequent heart looping after incubation. In the majority of cases, a right-sided heart loop was produced. He concluded that at stage 5 the intrinsic properties of the areas of precardiac mesoderm which affected the direction of looping had not yet been determined. Hoyle *et al.* (1992) in a similar series of studies, but including stage 6 embryos, reported that an intrinsic change occurs in the precardiac mesoderm between stages 5 and 6 that later influences the direction of looping of the tubular heart. Stage 6 appears as a key developmental transition period for the bilateral heart forming areas where a number of critical changes take place in the avian embryo (Linask and Lash, 1986; Linask, 1992a,b; Linask and Lash, 1993; Linask and Gui, 1995).

Stable commitment of precardiac cells occurs concomitantly with pericardial cavitation between stages 6 and 8 that delineates for the first time the cardiac cells as a separate compartment in the embryo (Linask, 1992a; Linask and Gui, 1995). The cardiac cells undergo a mesenchymal to epithelial cell shape change and acquire polarity. Epithelialization was suggested by our earlier cited work to be the first phenotypic indicator of stable commitment of the cardiomyocyte. As the cardiac epithelium is formed, it begins to bend to form two endocardial tubes which fuse anteriorly at the embryonic midline during stage 8, at approximately 26–29 hr. The endocardial tubes continue to fuse in a cephalocaudad progression to form a single heart tube composed of an outer myocardium and an inner endocardium. These two layers of the heart are separated by an extended extracellular basement membrane type matrix, termed the cardiac jelly (Manasek, 1977). Bending or looping of the single heart tube to form a C-shaped tube is apparent in embryos of 30 hr and becomes more discernible, until by 40 hr the ventricular region of the heart lies well to the right of the embryo's body (Patten, 1971). In normal heart development looping is an essential process which aligns the different chambers of the heart in their correct positions with the atria located above the ventricles. The heart is also aligned for the subsequent formation of septa, the outflow tract, and the connecting vasculature of the heart.

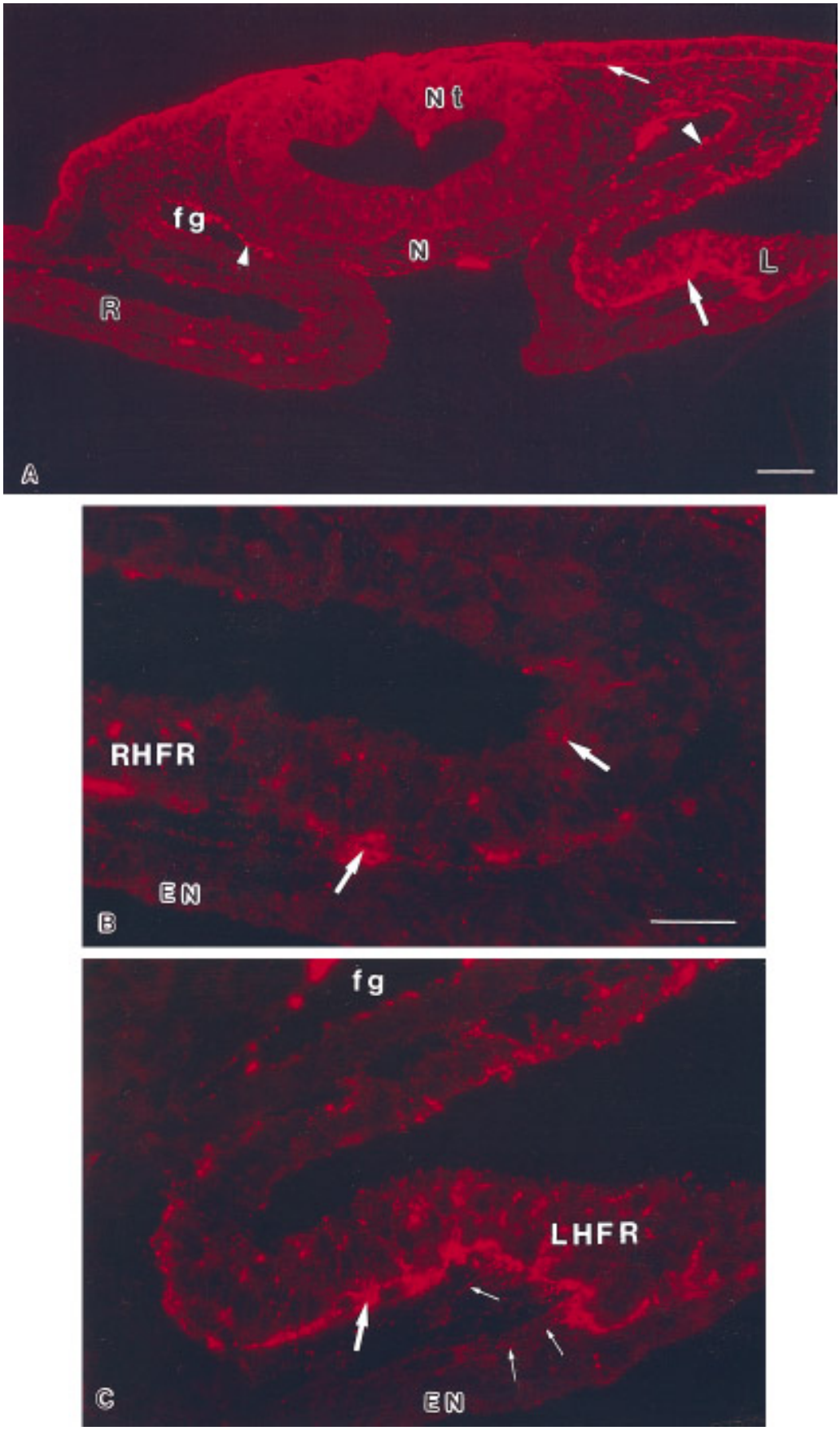
In this study we report on an asymmetrical localization of a specific extracellular matrix protein that is expressed earlier than stage 7+ (3 somites) in the heart forming region and that apparently relates to heart looping. This 250,000 M_r protein was originally extracted from the extracellular matrix of the developing eye in the chick embryo (Mieziewska *et al.*, 1994a). Immunoprecipitation of flectin (F-22 antigen) from Day 14 embryonic hearts shows the same single band as extracted from the eye that migrates at 250,000 M_r. Flectin (F-22) antibody was found to localize in a specific spatiotemporal manner to a number of regions in the chick embryo, including the eye, gut, and heart. The initial characterization of this protein and embryonic ocular localization in later stages has been reported separately in the chick and mouse (Mieziewska *et al.*, 1994a,b; Philip *et al.*, submitted for publication). The characteristics of flectin antigen do not match any known matrix proteins. It appears not to be a collagen type molecule based upon its amino acid composition and enzymatic degradation characteristics. Evidence suggests that in the eye it may be binding to chondroitin sulfate. Binding data of flectin to heparin Sepharose resins also support a possible ionic interaction of flectin with other matrix components.

During avian heart development, flectin appears to be modulated by retinoids, directly or indirectly, as deduced from a general reduction and lack of organization of the extracellular matrix components, as well as of flectin, comprising the cardiac jelly of vitamin A-deficient quail embryonic hearts. Hearts of vitamin A-deficient quail embryos do not loop and 65% show abnormal positioning on opposite side of the midline to that seen in normal embryos (Twal *et al.*, 1995). In addition, one very occasionally observes in normal embryos a heart that has spontaneously looped to the left. In one such embryo, flectin expression was seen predominantly on the opposite side, i.e., on the right, and reduced in intensity of immunolocalization of F-22 antibody from that normally observed.

MATERIALS AND METHODS

Embryos. White Leghorn chick embryos were obtained for immunohistochemistry at the various stages indicated

FIG. 1. Immunofluorescence micrographs of flectin in bilateral heart forming regions of the stage 8 chick embryo. (A) Flectin is seen predominantly on the left side (L) throughout the committed cardiac mesoderm with increased expression on the basal side of the mesoderm (large arrow) at the mesoderm–endoderm interface. This increased localization at the interface is in the same region where we previously reported fibronectin fibril deposition (Linask and Lash, 1986) and where cytotactin also localizes (unpublished observation). Flectin expression is also apparent with equal intensity in the anterior foregut (fg) on both sides of the midline (white arrowheads). Localization in the foregut region is the first area where flectin is observed in the early stage 7 embryo, preceding the expression seen in the bilateral heart forming regions. Flectin expression is also apparent in the basement membrane underneath the ectoderm primarily on the left side initially (small arrow). (B and C) Higher magnification of above flectin protein localization in right heart forming region (RHFR) in (B) compared with the left heart forming region (LHFR) in (C). On both sides flectin localizes to the extracellular matrix, as well as to cell surfaces of the cardiomyocytes. In an area of the cardiac epithelium that soon will bend in to form the endocardial tube, flectin also appears to be associated with small fibrils extending from the mesoderm toward the endoderm on the left side (small arrows in C). N, notochord; Nt, neural tube. Magnification bars: A, 50 μm; B and C, 20 μm.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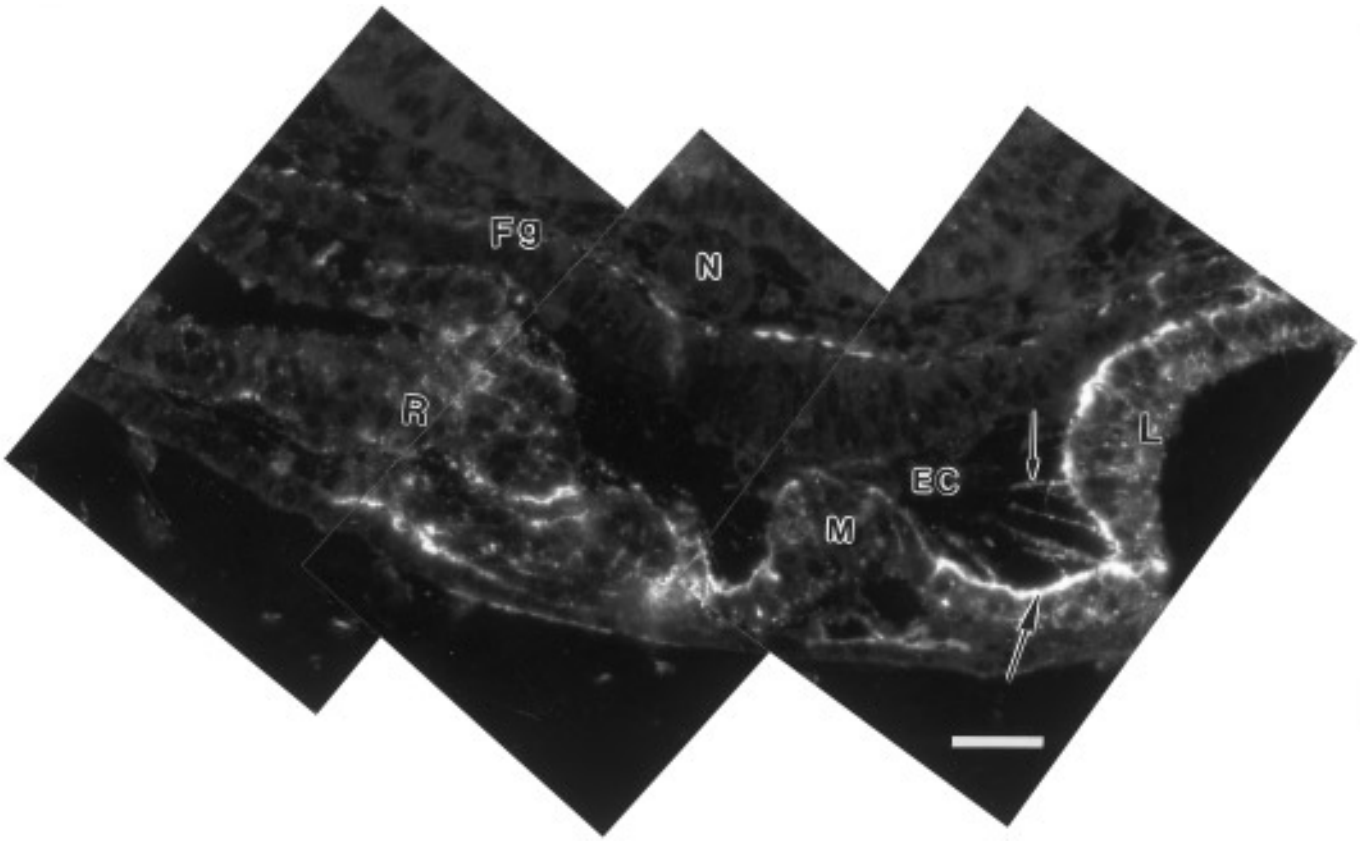


FIG. 2. Flectin expression in a cross section of a stage 9 embryo where the anterior heart forming regions have already fused to form a single tubular region. Fibrils associated with flectin (small arrow) extend in toward the developing endocardium (EC) in a region where the cardiac jelly is beginning to be apparent. Flectin staining is predominantly present in the matrix of the myocardial basal lamina on the left side (large arrow). Flectin continues to stain the foregut (Fg). R, right; L, left; Fg, foregut; N, notochord. Magnification bar, 15 μm.

within the text. Staging is according to Hamburger and Hamilton (1951). Removal of embryos from yolk has been previously described in detail (Linask, 1992a). Similar stage normal quail embryos (*Coturnix coturnix japonica*) were also utilized. The vitamin A-deficient quail embryos were provided by Dr. Maija Zile. The breeding and characterization of vitamin A-deficient embryos has been described previously (Dersch and Zile, 1993; Twal *et al.*, 1995).

Fixation and immunostaining. A total of 28 normal avian embryos and 8 vitamin A-deficient embryos (3–7 embryos of each stage shown) were first immunostained, then embedded in plastic, sectioned, and examined through the heart forming region. Only one normal embryo *in ovo* was obtained during the course of this study that showed a heart that had spontaneously looped to the left. The flectin distribution in this embryo is shown in Fig. 7A.

The immunohistochemical procedure has been described in detail in earlier publications (Linask, 1992a). Briefly, the blastoderms were removed from the yolk in phosphate-buffered saline and rinsed well. They were then transferred to Histochoice (Amresco, Inc., Solon, OH) for fixation for 1 hr at room temperature. PBS rinses followed with permeabilization of the embryos carried out using 100% methanol at −20°C for 1 hr. Rehydration was done through a graded alcohol series back to PBS. The last 30-min rinse was in PBS containing 1.0% albumin. Primary antibody incubation with mouse F-22 monoclonal antibody made against flectin was used overnight at 4°C. This was followed by three 30-min rinses in PBS–albumin. The embryos were then transferred into Texas red-conjugated rabbit antibody to mouse IgG (Organon Teknika Corp., Durham, NC) for overnight incubation at 4°C. Following subsequent rinses, the embryos were marked with carbon particles for orientation during sectioning. Subsequently, the embryos were dehydrated in a graded ethanol series to 100% ethanol, embedded in araldite, and sectioned at 2 μm through the heart areas.

Microscopy. Observations for immunohistochemical localization of flectin were made with a Nikon Optiphot-2 epifluorescence microscope equipped with the episcopic-fluorescence attachment EFD-3. Photographs were taken with black and white Kodak Tmax ASA 400 film or Kodak Ektachrome color film ASA 400.

Flectin antibody (F-22). The mouse monoclonal antibody F-22 was characterized and provided by Dr. Nancy

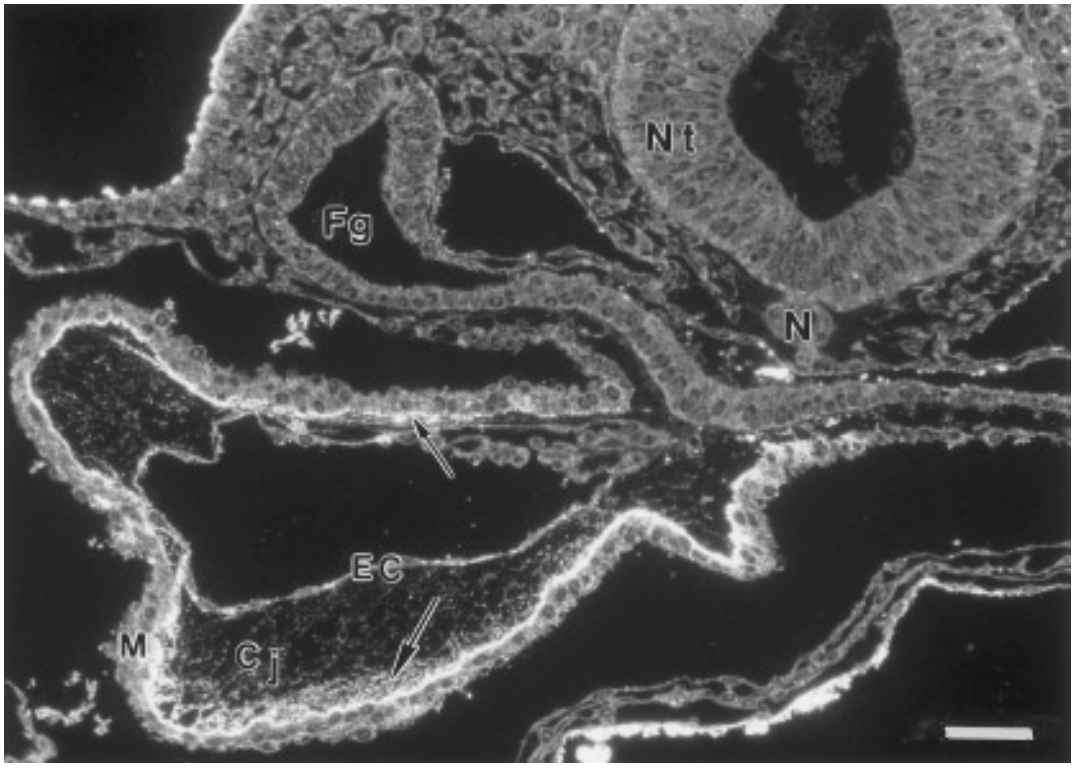


FIG. 3. Flectin localization in the looping heart at HH stage 14 (50–53 hr of development). Flectin maintains its asymmetric localization. It localizes at high levels predominantly on the outer, convex side of the looping heart; in the extracellular matrix of the myocardium (M), in the basal lamina of the myocardium (large arrow), in the cardiac jelly, and on endocardial cells (EC). A lattice-like fibrillar localization is seen within the cardiac jelly (Cj) extending from a high level of expression underneath the myocardium in a gradient toward the endocardium which also reacts with flectin antibody. On the concave side of the heart closer to the right foregut region, the cardiac jelly is reduced in amount (small arrow). Here flectin localizes to the basal lamina of the myocardial wall, but in reduced intensity to that seen in the convex side. Localization on the outer ectoderm of the embryo and endoderm of splanchnopleure is usually apparent, possibly due to nonspecific absorption of flectin antibody to outer embryonic surfaces. Nt, neural tube; N, notochord; Fg, foregut. Magnification bar, 50 μm.

Philp (Pennsylvania College of Optometry, Philadelphia, PA). The immunological specificity and characterization of the antibody and antigen have been reported separately (Mieziewska *et al.*, 1994a,b; Philp *et al.*, submitted for publication).

RESULTS

The localization of flectin in normal avian embryonic hearts between stages 7–22 and Embryonic Day 10 shows a changing pattern of expression during looping and in later hearts. The pattern as it relates to avian heart development is described here. Localization of flectin in other regions of the embryo and specifically in the mouse will be described and discussed separately (manuscript in preparation).

In the figures, where necessary, the right side of the heart and embryo is indicated with a R; the left side with a L. The basal side of the myocardium refers to the side facing the cardiac jelly; the apical side faces the pericardial coelom. Convex side of the heart refers to the outer bend or curvature of the heart; the concave side is situated close to the developing foregut region of the embryo.

Precardiac Epithelialization Stages

Flectin is not observed in the precardiac regions prior to epithelialization. The first appearance of flectin in the heart forming region occurs approximately at the 2- to 3-somite stage. A majority of the cells in the cardiac region along the cephalocaudad axis have become stably committed by this time point (Linask and Gui, 1995). Flectin is expressed after pericardial coelom formation in the extracellular matrix (ECM) of the committed cardiac mesoderm predominantly in the left heart forming region (LHFR). Less flectin expression is apparent in the ECM of the right side. The apparent delay of expression in the right side leads to an asymmetric distribution, as seen in a cross section through the heart forming region at stage 8+ (5 somite) embryo (Figs. 1A, 1B, and 1C). At stage 8+ as the left cardiac epithelium is just

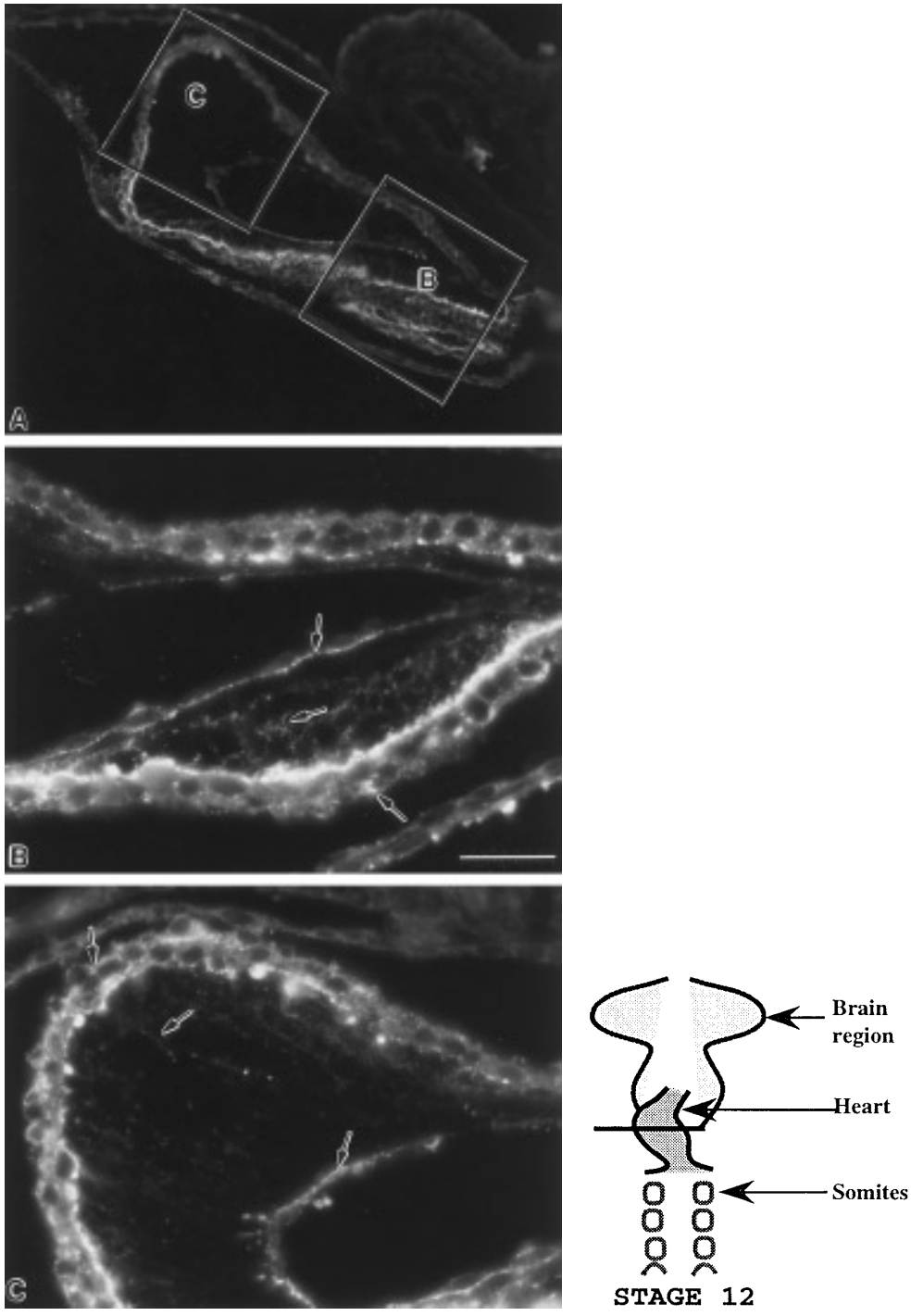
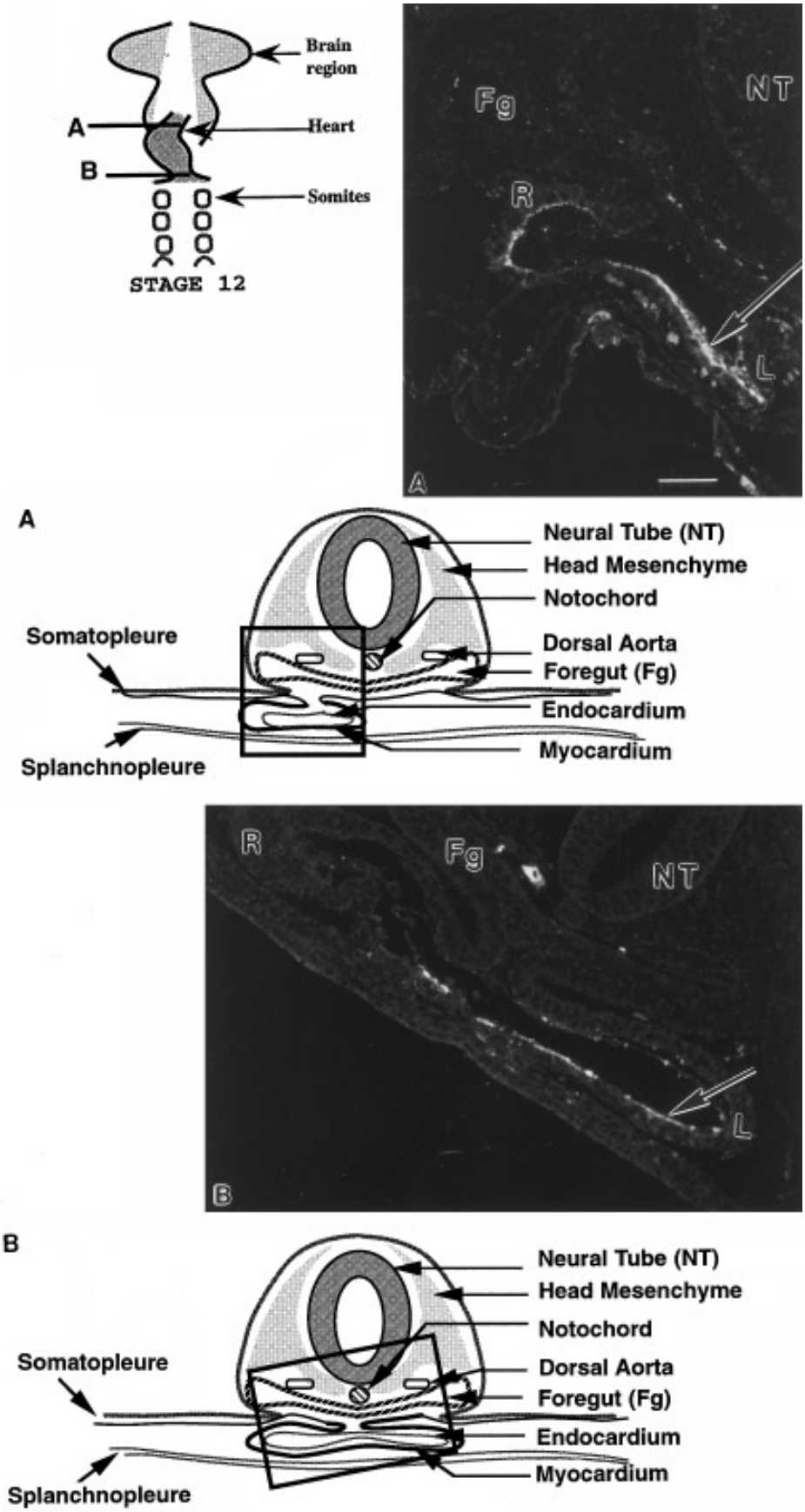


FIG. 4. Differential organization of flectin in the same plane of a HH stage 12 looping heart (45–49 hr of development). (A) Orientation of micrographs B and C, shown at higher magnification. Schematic drawing shows approximate level of section. (B) An area on the outer convex side. Here the flectin is organized in a lattice-like arrangement which is also apparent as one focuses up and down in this area. In C, a different arrangement of flectin is apparent with a fine fibrillar array of flectin extending radially from the myocardium to the endocardium which also localizes F-22 antibody. In general the myocardial cells are associated with flectin. Small arrows point to flectin localization throughout these areas. Magnification bar in B and for C, 30 μm.


Brain region
A
Heart
B
Somites
STAGE 12
Fg
NT
R
L
A
A
Neural Tube (NT)
Head Mesenchyme
Notochord
Somatopleure
Dorsal Aorta
Foregut (Fg)
Endocardium
Myocardium
Splanchnopleure
R
Fg
NT
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Neural Tube (NT)
Head Mesenchyme
Notochord
Somatopleure
Dorsal Aorta
Foregut (Fg)
Endocardium
Myocardium
Splanchnopleure

beginning the process of forming a tubular structure (Fig. 1C), flectin-associated fibrils are seen extending from the basal side of the differentiating myocardium toward the endoderm (EN; see small white arrows). This organization is maintained as the lateral sides of the cardiac mesoderm bend to form the heart tube (Fig. 2, see arrows). A few endocardial cells (EC) are apparent in this figure.

Later Stages of Heart Development

At HH stage 12–14 (between 45 to 53 hr of development) localization of flectin at the level of bending is predominantly on the outer convex side of the tubular heart, on the basal aspect of the myocardium, and within the cardiac jelly extending in a gradient to the endothelial cells (EC) which also express flectin (see Figs. 3 and 4). In different regions of the cardiac jelly, but in the same plane along the anterior–posterior axis of the tubular heart, differential organization of flectin is apparent. This is shown in Figs. 3 and 4A–4C, where the localization in the outer curvature (Fig. 4B) is contrasted with that at the end of the loop (Fig. 4C). In the convex side, flectin is organized more in a lattice-like fashion near the myocardium; while at the outer loop, it extends in toward the endocardium in a finer fibrillar array (Fig. 4C). Whether the fibrils actually attach to the endocardium in all areas is not completely clear, although at some levels of the heart tube this appears to be the case (e.g., Figs. 3 and 7). At more anterior and posterior levels the localization also changes in comparison to the bending or looping region. In Fig. 5A flectin expression localizes in the anterior tubular region to the basal side of the myocardium. In Fig. 5B a section through a posterior region also shows the basal localization. The left–right asymmetric distribution is maintained throughout the heart tube.

In comparison to the earlier described stages of 12–14 shown above, there is a diminished expression of flectin in the cardiac jelly at stage 22 or approximately at 3.5 days of development (Fig. 6A). Fine fibrillar material is still apparent, but much reduced in intensity of immunostaining. On Embryonic Day 10 of heart development flectin is again expressed in a patch-like pattern in the extracellular matrix of the myocardium (arrow) and perivascular lining (white arrowhead, Fig. 6B) and also in the valvular matrix (arrow in Fig. 6C).

Occasionally one observes a normal embryo which has a heart that has abnormally looped to the left. The heart from one such stage 12 quail embryo (17 somites) displayed a reduced, as well as abnormal, flectin expression predominantly in the right side of the heart associated with the myocardium and endocardium with little in the cardiac jelly (Fig. 7A). Compare with immunolocalization seen in a normal avian embryo at a comparable stage in Fig. 4). In addition, vitamin A-deficient quail embryos develop thin-walled, dilated, and nonlooping hearts (Heine *et al.*, 1985; Dersch and Zile, 1993). One such vitamin A-deficient embryo is shown in cross section at low magnification on Day 2 of development (45–49 hr incubation; see Figs. 7B and 7C). Area boxed in a region in Fig. 7B is shown at higher magnification in Fig. 7C. Arrows in Fig. 7C point to reduced flectin distribution in a disorganized cardiac jelly, compared to normal embryos. Note also in vitamin A-deficient embryos the absence of flectin on the basal side of the myocardium, which stains heavily in normal avian embryos at a comparable time of incubation between 45 and 53 hr (cf. Figs. 3 and 4). More posteriorly in the vitamin A-deficient hearts, the cardiac jelly is absent with no flectin discernible (not shown).

DISCUSSION

Flectin, a novel protein first characterized in eye development, localizes in an asymmetric fashion to different compartments of the developing early heart and may be associated with the looping process. Our observations indicate that the left–right asymmetry which emerges early in the embryo during the gastrula stages (Wolpert, 1989) may in the heart region be associated with a retinoid-modulated pathway affecting extracellular matrix synthesis that includes regulating flectin expression first in the left heart forming region and slightly later in the right side. This asymmetric delay in flectin expression leads to a left/right asymmetry that is maintained throughout the looping stages. The asymmetry in F-22 antibody localization is seen in the developing heart, but not in the developing foregut tube, which is another area of increased localization throughout these early stages.

In the vitamin A-deficient embryonic hearts, most of the hearts reveal a dilated appearance and fail to loop normally and the tubular hearts are positioned on the left side of the midline (Twal *et al.*, 1995). In these dilated hearts, there is often a decreased amount of cardiac jelly compared to normal quail hearts. This apparently relates to a generally decreased synthesis of cardiac jelly extracellular matrix com-

FIG. 5. Flectin localization at anterior and posterior levels of the heart tube during looping of a stage 12 embryo (45–49 hr of development). Approximate levels of the heart in A and B are indicated in accompanying schematic diagram. For orientation, labeled schematic diagrams are also included for each section with the boxed in region indicating area shown in figure. (A) A section through an anterior most heart region. Flectin is apparent (arrow) primarily on the left (L) basal aspect of the tubular myocardium on the developing convex side of the heart tube. In B at the posterior level of the tubular heart, flectin remains discernible primarily on the basal aspect of the myocardium (arrow). Flectin is reduced in the amount of expression and localization in comparison to the immunostaining pattern seen in more anterior and bending regions of the heart tube. Compare with Fig. 4. Left (L)/right (R) asymmetry is maintained throughout the heart tube. NT, neural tube; Fg, foregut. Magnification in A and for B, 50 μm.

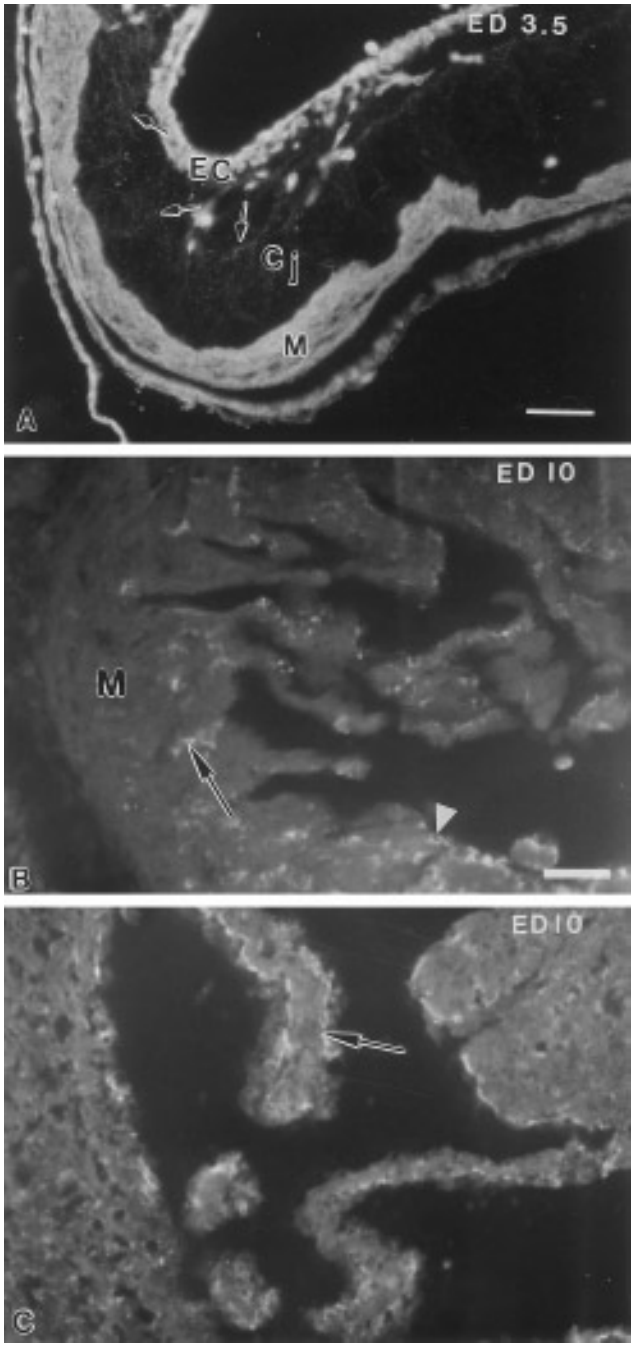
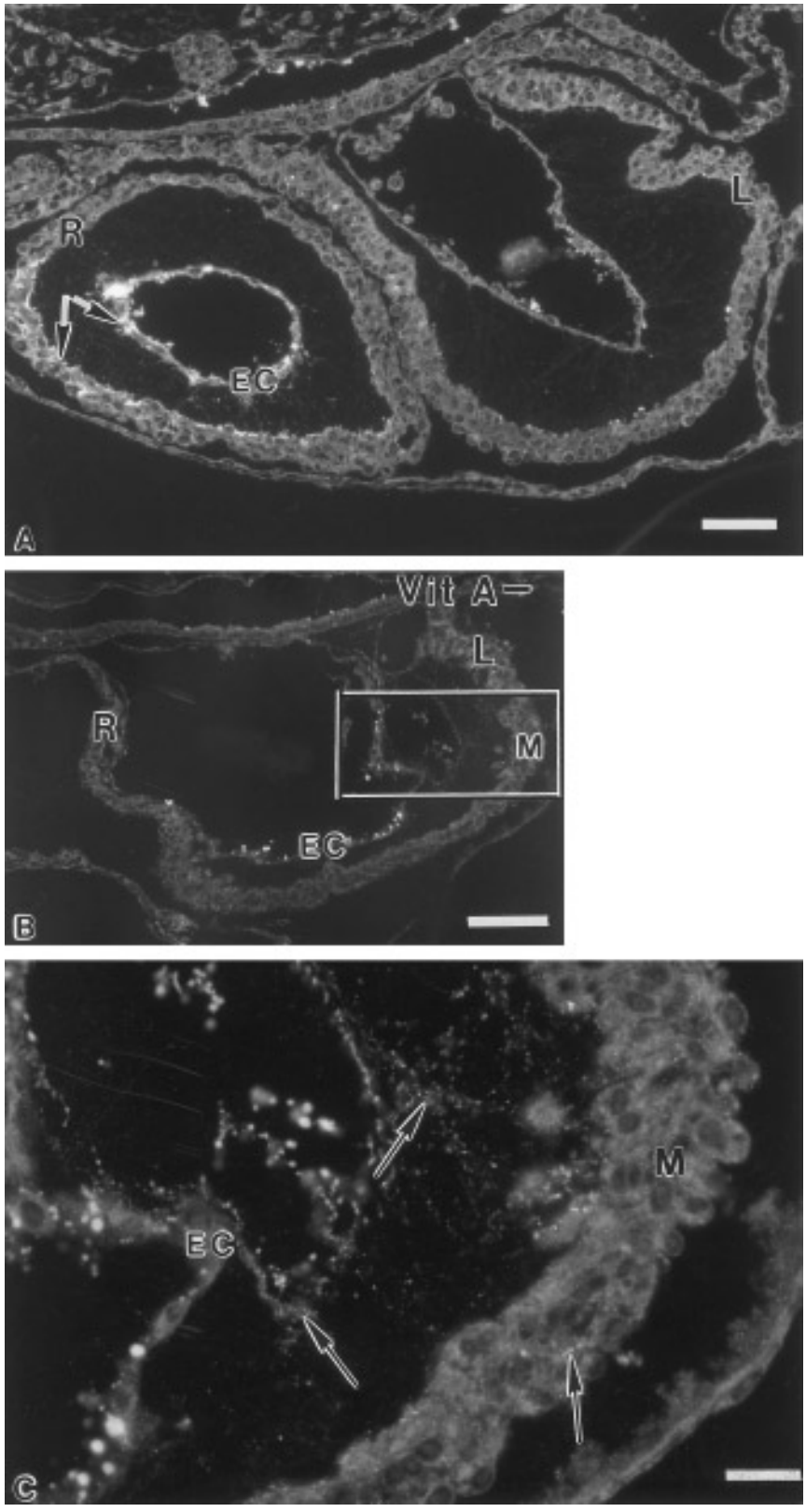


FIG. 6. A comparison of flectin localization in the heart at stages corresponding to Embryonic Day (ED) 3.5 and ED 10. (A) At ED 3.5 (HH stage 22) at the level of the looped part of the heart localization of flectin (small arrows) is greatly decreased within the cardiac jelly (Cj) and little left/right asymmetry is appreciable. In comparison to earlier immunostaining patterns (cf. Figs. 3 and 4), in this older embryo flectin is essentially absent from the myocardium (M) and its basal layer and from within the cardiac jelly. A fine punctate pattern of immunostaining as shown here (small arrows) may be observable in the cardiac jelly and associated with the endocardium (EC). Magnification bar, 30 μm. (B and C) In ED 10 chick hearts (cryosectioned for better antibody penetration in older stage hearts) and stained, flectin is again expressed in the myocardium (M; see arrow), as well as in the endocardial lining in the perivascular areas (white arrowhead). In C flectin is present in the valvular connective tissue (arrow) as well as in perivascular areas. At these later developmental stages, i.e., after 3 days, left/right asymmetry can no longer be observed in flectin localization. Magnification bar for B and C, 15 μm.

ponents. Whether the retinoid effect is direct or indirect through the modulation of growth factors remains to be investigated. It is interesting to note that in a recent report a linkage is made in *Xenopus* between dorsal-anterior development involving the organizer region and cardiac left–right asymmetry (Danos and Yost, 1995). In the chick the Hensen's Node region which is the equivalent of the *Xenopus* organizer is known to be enriched in retinoic acid (Chen *et al.*, 1992). In the latter cited study, it is suggested that the endogenous retinoids may establish a concentration gradient from the Henson's Node to adjacent tissues. Experimental manipulations of the Node and primitive streak regions indicate that during gastrulation precardiac cells do not move through the Node, but immediately caudal to it (Garcia-Martinez and Schoenwolf, 1993; Inagaki *et al.*, 1993). Thus, during gastrulation the precardiac cells may be exposed to a gradient of retinoic acid as they move through the primitive streak laterally to form the mesoderm within the bilateral heart forming areas. Between stages 5 and 8 there is an increasing diffuse localization of all-*trans* retinoic acid in the heart forming regions (Twal *et al.*, 1995). The mRNAs for retinoic acid receptor RARβ2 in these bilateral cardiac regions are expressed in a gradient with increasing amounts in the posterior endoderm and precardiac mesoderm within the heart regions (Kostetskii *et al.*, 1995). Whether retinoic acid may be exerting its effects indirectly in modulating extracellular matrix components, including flectin, as early as avian gastrulation needs to be determined.

Recent studies show that specific genes relating to the activin receptor IIa, sonic hedgehog, and cNR-1 (nodal) are also expressed asymmetrically during and after gastrulation and regulate each other's expression in a sequential pathway (Levin *et al.*, 1995). Manipulation of the expression of these genes alters heart situs. It could follow that the normal regulation of the above genes may lead downstream to an asymmetrical distribution of flectin expression. Therefore, flectin may serve as a marker that would allow a direct molecular analysis of the activin-dependent signaling pathway preceding a specific asymmetric protein expression pattern which affects subsequent heart looping.

Flectin appears as a component of the heart extracellular matrix complex. The extracellular matrix of the myocardium, the cardiac jelly, and its extension to the endocardium appear to be important in the looping process. The structure of the extracellular matrix complex of the heart exhibits a high degree of organization (Nakamura and Manasek, 1978a,b). Flectin is associated with cells of the myocar-



dial layer, but whether it interacts with specific cell membrane receptors is currently unknown. As perturbation of actin also perturbs looping (Itasaki, 1991), the possibility exists that a molecular cross-bridge may exist between flectin and the cytoskeleton. Regional microheterogeneity of flectin is observed at the basal side of the myocardium, within the cardiac jelly, and in relation to the endocardium. During looping between stages 12 and 14 the highest level of expression and organization of flectin appears to be in the outer convex side. A high intensity of expression is observed throughout the myocardial wall and extends in a lattice-like organization through the cardiac jelly to the cell surfaces of the endocardial cells. In the outer loop area, flectin is organized in association with fine fibrils extending radially from the myocardium toward the endocardium. In some regions a direct association of fibrils extending from the myocardium to the endocardial cells is not definite, although the endocardial cell surfaces show flectin localization (e.g., Fig. 4B). Whether the fibrils are composed of flectin is not known. The fibrils may be another molecular component, since often in areas of weaker immunostaining, the staining pattern is intermittent along a fibril. The lower levels of flectin and its radial organization seen here in the lateral bulging loop suggest that this pattern may be involved in providing a microenvironment of greater elasticity and compliance, allowing for the thin myocardial wall to push out. In addition, different levels along the anterior–posterior axis of the heart tube show differential localization. Anteriorly and posteriorly along the heart tube away from the pronounced looping region, the localization is reduced in intensity and limited to only the basal aspect of the myocardium, although asymmetry is maintained. Conceivably changes in composition and structural organization of the extracellular matrix components as flectin along the different axes and planes of the heart tube may result in the necessary local tensions to affect heart looping.

Flectin may interact with other extracellular matrix components. Regions of high levels of flectin expression, such as at the mesoderm–endoderm interface at stage 8 and at the myocardial basal lamina at stages 12–14, correspond to fibronectin (FN) expression which, however, is localized symmetrically (for FN localization at early stages see Linask and Lash, 1986; for older stages see Icardo and Manasek, 1983; Mjaatvedt *et al.*, 1987). The cardiac jelly in which flectin is present in a highly organized manner does not appear to localize fibronectin antibody except at very specific regions (Icardo and Manasek, 1983). Fibronectin involvement with asymmetrically localized molecules, however, is implied in that the perturbation of the extracellular matrix by the microinjection of RGD containing peptides into the blastocoele of *Xenopus* embryos resulted in global randomization of left–right asymmetries (Yost, 1990, 1992). Similarly, in our previous studies on heart development perturbation of cell–fibronectin interactions using fibronectin antibodies or RGD-synthetic peptides, randomized looping or cardiabifida was also observed (see Linask and Lash, 1988). Preliminary perturbation experiments using F-22 antibody during whole embryo incubation indicates cardiabifida often occurs with no looping (manuscript in preparation). Flectin in the eye is closely associated with chondroitin sulfate glycosaminoglycans (Mieziewska *et al.*, 1994a). The purified flectin antigen is resistant to digestion with chondroitinase ABC lyase, hyaluronidase, keratinase, and collagenase (Philp *et al.*, submitted for publication). It is suggested that during heart development, flectin by its possible interaction with other extracellular matrix components which may be symmetrically expressed may induce the tensions necessary to affect directional looping. Conceivably, the perturbation of any of the extracellular matrix components which flectin may be interacting with (cf. perturbation of fibronectin using RGD-synthetic peptides), or perturbation of flectin itself, would lead to the noted looping abnormalities.

Flectin is expressed at key developmental stages in different matrix compartments of a number of vertebrate species. It is developmentally regulated, but not exclusively, in a number of structures that form tubes or bend or loop in some manner as in the eye, heart, foregut, somite, and kidney. From a comparison of the distribution of flectin antibody in the interphotoreceptor matrix (IPM) of vertebrate species ranging from the human and mouse to zebrafish and frog, the antibody labeled the IPM of all 11 vertebrate species examined (Mieziewska *et al.*, 1994b). Flectin is also present in the matrix of the *Drosophila* compound eye. This

FIG. 7. Abnormal flectin staining in normal quail embryos with hearts that have looped abnormally to the left (Fig. 7A) or (Figs. 7B and C) in Vitamin A-deficient (Vit A-) quail embryos with hearts that do not loop. Fig. (7A). Occasionally one obtains normal embryos with hearts that spontaneously loop to the opposite side. One such quail embryo corresponding to HH stage 12 (17 somites) was immunostained with flectin antibody. Flectin localization (arrows) in the looped heart is seen reduced in amount and is now seen predominantly on the embryonic right side of the heart which is opposite to that observed in the normally looped heart (compare with immunostaining in Fig. 4 of a comparable stage embryo). Flectin is apparent on the right side (arrows), although its localization is much reduced in the cardiac jelly, as well as in the basal lamina of the myocardium and in the endocardium (EC). Flectin expression is absent on the left side (L). Magnification bar = 50μm. Fig. (7B). Vitamin A-deficient quail embryos develop thin-walled, dilated, and nonlooping hearts as shown here at low magnification on day 2 of development. These hearts show weak flectin expression within a greatly reduced, disorganized, cardiac jelly. The little cardiac jelly that is evident is primarily on the left side. Compare with flectin localization in the normal avian heart at a similar developmental stage (cf., Figs. 3 and 4). Magnification bar = 85μm. Boxed in region in this micrograph is shown at higher magnification in Fig. (7C). Arrows point to reduced flectin distribution in the myocardium, as well as in the reduced and disorganized cardiac jelly, as compared to that seen in normal embryos. Note also absence of flectin on basal side of myocardium that stains heavily in normal avian embryos (cf., Figs. 3 and 4). M, Myocardium; EC, endocardium. Magnification bar = 15μm.

indicates that flectin is evolutionarily highly conserved and must play an essential role in normal development.

ACKNOWLEDGMENTS

This research was supported by a National Institutes of Health Research Grant (HL48746) to K.K.L., a grant from the National Eye Institute (EY 05508) to N.P., and by U.S.D.A. Grant 91-37200-6285 and the Michigan Experimental Station (MHZ). T.T. is supported by the Noguchi Medical Research Institute, U.S.A. The technical support of Y. H. Gui and K. Liisi Linask is greatly appreciated.

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Received for publication July 21, 1995
Accepted October 18, 1995