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Expression of the *Mf1* Gene in Developing Mouse Hearts: Implication in the Development of Human Congenital Heart Defects

RUTH E. SWIDERSKI,[1] REBECCA S. REITER,[2] DARRYL Y. NISHIMURA,[1] WALLACE L.M. ALWARD,[3] JEFFREY W. KALENAK,[4] CHARLES S. SEARBY,[1] EDWIN M. STONE,[3] VAL C. SHEFFIELD,[1,5] AND JIM JUNG-CHING LIN[2]*

[1]*Department of Pediatrics, University of Iowa, Iowa City, Iowa*
[2]*Department of Biological Sciences, University of Iowa, Iowa City, Iowa*
[3]*Department of Ophthalmology, University of Iowa, Iowa City, Iowa*
[4]*Department of Ophthalmology, Medical College of Wisconsin, Milwaukee, Wisconsin*
[5]*Howard Hughes Medical Institute, University of Iowa, Iowa City, Iowa*

ABSTRACT **The transcription factor *FKHL7* gene has recently been associated with the anterior segment dysgenesis disorder of the eye known as Axenfeld-Rieger anomaly (ARA). A growing body of evidence indicates that mutations in *FKHL7* cause not only defects in the anterior segment of the eye but defects in the heart valves and septa as well. In order to evaluate its contribution to normal heart septation and valve formation, expression of the mouse homologue *Mf1* in embryonic hearts was analyzed by in situ hybridization. A weak but significant level of *Mf1* expression could be detected in the endocardium of mouse embryos as early as day 8.5 post-conception (p.c.). *Mf1* expression was undetectable in the hearts of day 9.5 p.c. embryos, but by day 10.5–11 p.c., *Mf1* transcripts could be found again in the endocardium of both the atrium and ventricle and a relatively strong signal was observed in the dorsal portion of the septum primum, in what appeared to be the spinal vestibule. At day 13 p.c. when aortic and pulmonary trunks are separated, relatively more *Mf1* transcripts were detected in the leaflets of aortic, pulmonary, and venous valves, the ventral portion of the septum primum, as well as in the single layer of cells on the edges of the atrioventricular cushion tissues. Surprisingly, there was no signal detected in the developing interventricular septum. At day 15 p.c., overall *Mf1* signals were greatly decreased. However, significant levels of expression could still be observed in the atrial septum, the tricuspid valve, the mitral valve, and in the venous valve but not in the interventricular septum. The temporal and spatial expression patterns of the *Mf1* gene in developing mouse hearts suggest that *Mf1* may play a critical role in the formation of valves and septa with the exception of the interventricular septum. This is further supported by our studies showing that mutations in the *FKHL7* gene were associated with defects in the anterior segment of the eye as well as atrial septal defects or mitral valve defects. *Dev Dyn 1999;216:16–27.***
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Key words: heart; forkhead/winged helix transcription factor; in situ hybridization; atrial septal defect; mitral valve defect; Axenfeld-Rieger anomaly

INTRODUCTION

Forkhead/winged helix proteins are a family of DNA-binding transcription factors that play key roles in embryogenesis, tissue-specific gene expression, and tumorigenesis (Kaufmann and Knochel, 1996). First identified as a homologous region in the *Drosophila* homeotic gene *forkhead* and in rat *hepatocyte nuclear factors 3* (*HNF3*) (Weigel and Jackle, 1990), the evolutionarily-conserved 110 amino acid DNA-binding domain has been found in widely diverse species ranging from yeast to human (Lai et al., 1991). This DNA binding domain, composed of a helix-loop-helix core flanked by two wing-like regions, interacts with its DNA target to form a 3D structure resembling the shape of a butterfly and is, therefore, called the winged helix motif.

We recently determined that mutations in the forkhead/winged helix family member *FKHL7* (for forkhead-like 7) cause a spectrum of anterior segment eye defects that are related to the development of congenital glaucoma associated with chromosome 6p25, most notably Axenfeld-Rieger anomaly (ARA) (Nishimura et al., 1998). ARA is an autosomal dominant ocular disorder characterized by anterior segment dysgenesis that presents with ocular features including a prominent, anteriorly displaced Schwalbe's line (posterior embryo-

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*Correspondence to: Dr. Jim Jung-Ching Lin, Department of Biological Sciences, 138 Biology Building, University of Iowa, Iowa City, Iowa 52242-1324. E-mail: Jim-Lin@Uiowa.Edu

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toxon) attached to strands of peripheral iris that bridge the iridocorneal angle, displaced pupils, and iris hypoplasia (Alkemade, 1969; Shields, 1983; Shields et al., 1985). These mutations are predicted to alter or disrupt the normal binding affinity of *FKHL7* and act in a dominant manner. Others have also recently identified *FKHL7* mutation-causing anterior segment defects of the eye (Mears et al., 1998).

Reports of patients with defects in the anterior segment of the eye in addition to a range of systemic abnormalities suggest that *FKHL7* mutations could also result in such systemic phenotypes as hydrocephalus, deafness, renal defects and congenital heart defects (Tsai and Grajewski, 1994; Cunningham et al., 1998; Law et al., 1998; Mammi et al., 1998). Consistent with this hypothesis is the demonstration that the *FKHL7* gene, also referred to as *FREAC3* (for forkhead-related-activator 3)(Pierrou et al., 1994) and the murine homologue *Mf1* (for mesoderm/mesenchyme forkhead 1) (Sasaki and Hogan, 1993), also termed *Fkh-1* for forkhead homologue-1 (Kaestner et al., 1993), are widely expressed in adult human and mouse tissues including the eye, brain, heart, and kidney (Kaestner et al., 1993; Pierrou et al., 1994; Kaestner et al., 1996; Hiemisch et al., 1998a; Mears et al., 1998; Nishimura et al., 1998). Transcripts have also been detected in fetal human heart and kidney, in numerous embryonic mouse and chicken tissues, particularly those of mesenchymal origin, as well as in the endocardium of developing murine and avian hearts (Sasaki and Hogan, 1993; Pierrou et al., 1994; Buchberger et al., 1998; Hiemisch et al., 1998b; Kume et al., 1998; Nishimura et al., 1998). Heterozygous *Mf1* mutant mice have anterior segment eye anomalies similar to those seen in Axenfeld-Rieger anomaly patients (Mears et al., 1998), whereas homozygous null *Mf1* mice die perinatally with multiple abnormalities including skeletal malformations due to lack of differentiation of pre-chondrogenic mesenchyme into cartilage, and hydrocephalus caused by a failure of arachnoid cell differentiation into meninges (Kume et al., 1998). Their phenotype is identical to that of the spontaneous pleiotropic mouse mutant *congenital hydrocephalus* (*ch*), which has a stop codon within the *Mf1* forkhead/winged helix domain resulting in a truncated protein lacking the DNA-binding domain (Kume et al., 1998).

An increasing number of case reports shows the coexistence of congenital cardiac defects in patients with ARA and Axenfeld-Rieger syndrome (ARS) (Tsai and Grajewski, 1994; Cunningham et al., 1998; Law et al., 1998; Mammi et al., 1998). ARS is an autosomal dominant disorder that, in addition to ARA-like ocular disorders, is characterized by systemic abnormalities of the teeth, jaw, and umbilicus (Alward and Murray, 1995). The observation of dysgenesis of the anterior segment of the eye in combination with cardiac malformations in a subgroup of ARA and ARS patients may be coincidental, or alternatively, the ocular and cardiac anomalies may be the result of malfunction of an affected gene that contributes to certain ocular and cardiac developmental disorders of neural crest tissue (Tsai and Grajewski, 1994). Recent molecular studies have shown that some cases of ARS are caused by mutations of the *RIEG1/PITX2* gene at 4q25 (Semina et al., 1996) as well as a second locus (*RIEG2*) at 13q14 (Phillips et al., 1996), We have noted that several ARA patients with mutations in *FKHL7* have congenital heart disease (Nishimura et al., 1998). Although the small numbers of patients studied to date do not allow us to confirm the association statistically, it prompted us to evaluate the expression of the murine homologue of the gene, *Mf1,* in the developing mouse heart by in situ hybridization. The expression pattern at the transcript level is consistent with the hypothesis that *Mf1* plays a critical role in the formation of the atrial septum and cardiac valves in the developing heart.

RESULTS

Localization of *Mf1* Transcripts in Developing Embryos and Hearts by In Situ Hybridization

Previous studies of *Mf1* expression during early mouse embryogenesis first reported the presence of transcripts in the paraxial mesoderm at early gastrulation and in the presomitic mesoderm, somites, cephalic mesoderm, and first branchial arch of day 8.5 post-conception (p.c.) embryos (Sasaki and Hogan, 1993). We also observed a similar pattern of expression in day 8.5 p.c. embryos. In addition to the strong labeling of the somites (so) and mesenchyme around the pericardial peritoneal canal (ppc), weak but significant labeling of the endothelial lining (ec) of the heart tube was seen (Fig. 1A, B). Overall, expression of *Mf1* continued to increase until day 9.5 p.c. when signal intensity was greatly reduced. Very little, if any, signal was detectable in day 9.5 p.c. hearts (Fig. 1C, D). Diminished *Mf1* expression in the thoracic region of the day 9.5 p.c. embryo is in agreement with previous reports (Sasaki and Hogan, 1993; Kume et al., 1998), although the in vivo significance of this decrease remains unclear.

In order to correlate the potential function of Mf1 in the development of heart valves and septae, we analyzed the temporal and spatial expression of *Mf1* in day 10.5–11 p.c. to 15 p.c. embryos by in situ hybridization. It has been established that the formation of valves and septae takes place during this critical developmental period (Webb et al., 1998).

Day 10.5–11 p.c. Embryos

At this stage, the heart is still a single segmented looping tube, but at the atrioventricular canal (avc) region, a few invading mesenchymal cells can be seen, and in the middle of the atrium, the septum primum (sp) is readily detected (Figs. 2 and 3). When the embryo was cut in cross-section from cranial to caudal and analyzed by in situ hybridization with an *Mf1* antisense riboprobe, the majority of *Mf1* expression was

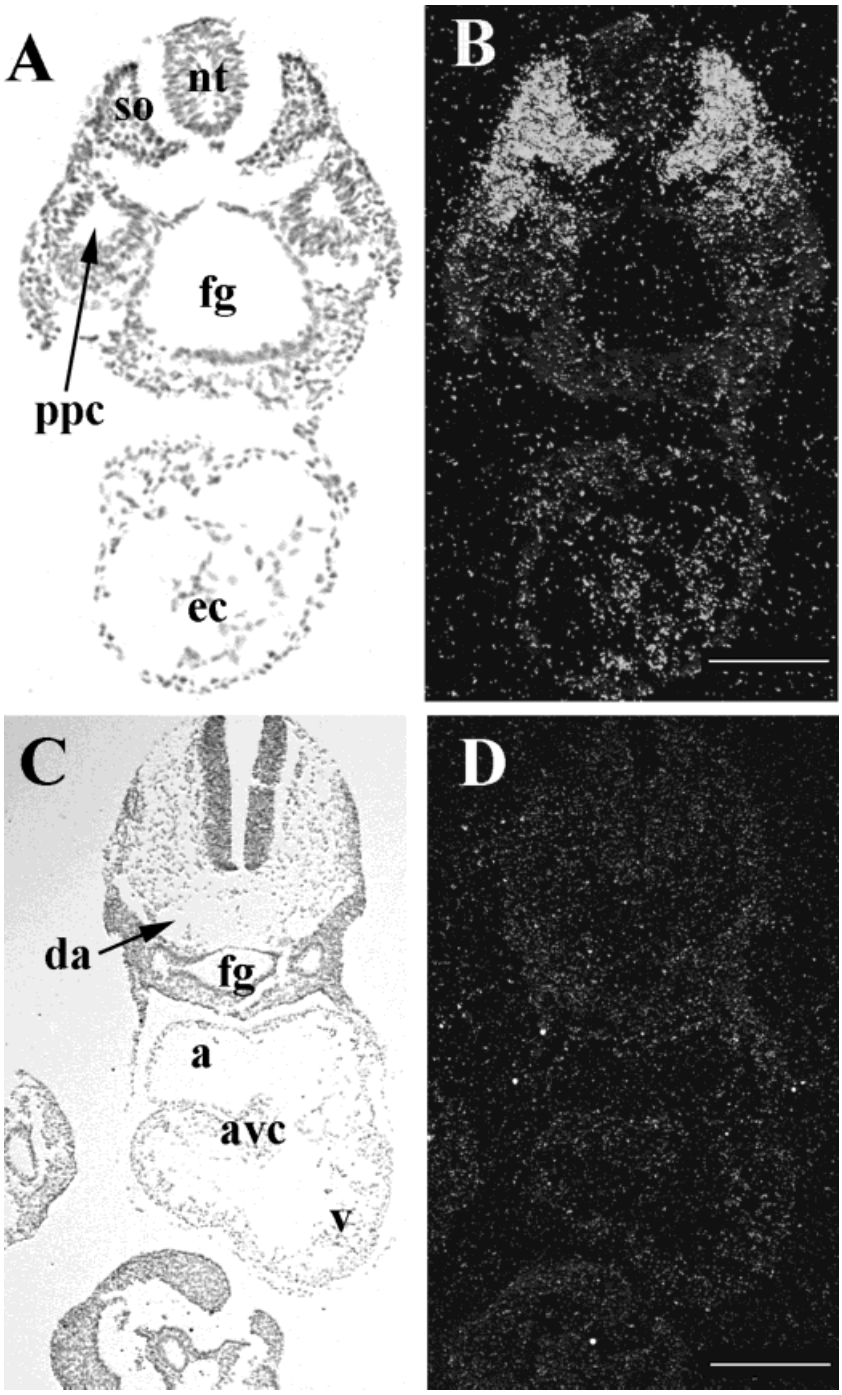

Fig. 1. Detection of *Mf1* transcripts in day 8.5 and 9.5 p.c. embryos by in situ hybridization. Representative 7-µm-thick cross-sections of murine embryos at day 8.5 (A, B) and 9.5 (C, D) p.c. were hybridized with antisense ^{35}S-labeled *Mf1* riboprobe. Specimens were visualized under bright-field optics for the identification of tissue structure (A, C) and the same field visualized under dark-field optics for the detection of hybridization signal (B, D). **(A, B)** Strong signal was detected in the somites (so) and the dorsal portion of mesenchyme around the pericardial peritoneal canal (ppc) of day 8.5 p.c. embryo. A weak but above background signal was also detected in the rest of mesenchymal tissues and the endocardium (ec) of the heart tube. neural tube (nt); foregut (fg). Bar = 115 µm. **(C, D)** Very little signal above background was detected. The background signal was determined by in situ hybridization on adjacent sections with *Mf1* sense riboprobe (data not shown). dorsal aorta (da); atrium (a); atrioventricular canal (avc); ventricle (v); foregut (fg). Bar = 290 µm.

localized in the somites, in the dorsal portion of the aortic sac (data not shown), in the mesenchyme surrounding the anterior cardinal vein (acv), the dorsal aorta (da), the foregut (fg), and the umbilical vein (uv) (Fig. 2). Cardiac tissue expression was predominantly in the dorsal component of the developing septum primum (sp) (Fig. 2). At higher magnification shown in Figure 3, a significant level of *Mf1* expression was detected in most of the inner layer of the atrial wall, which is continuous with a dense layer of mesenchymal tissue. Following examination of 34 consecutive sections (7 µ per section), we found that this *Mf1*-positive dense layer of tissue could be traced back to mesenchymal tissue that is continuous with that surrounding the laryngeo-tracheal groove (Figs. 3 and 4). Therefore, this extra-cardiac component of the septum primum most likely represents the tissue called spina vestibuli (Tasaka et al., 1996; Webb et al., 1998). To a lesser extent, *Mf1* was expressed in the trabeculated wall of the ventricle (Fig. 2). From 11 days p.c. onward, the

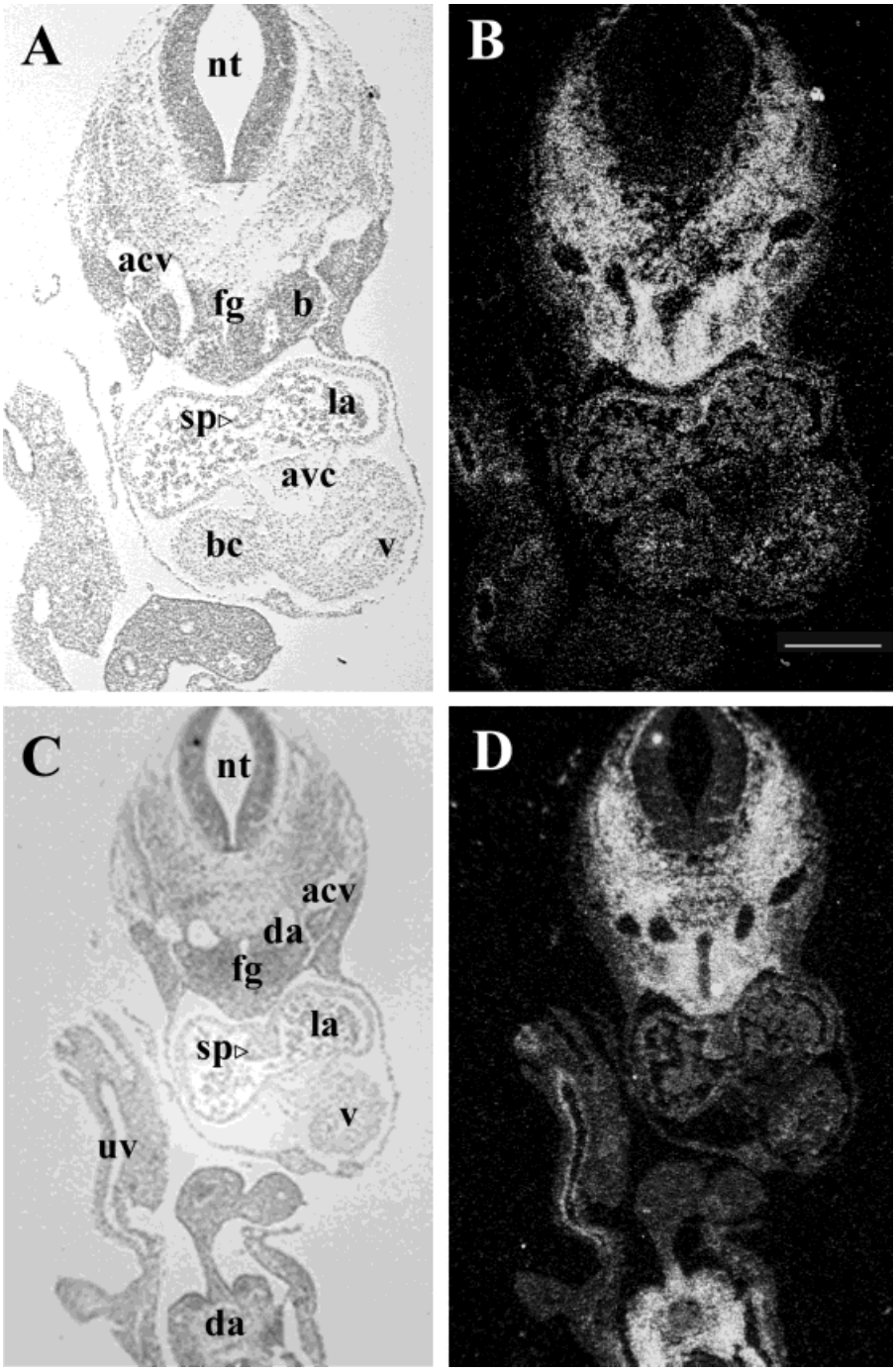

Fig. 2. Detection of *Mf1* transcripts in day 11 p.c. embryos by in situ hybridization. Antisense *Mf1* riboprobe was hybridized to embryo cross-sections and analyzed using bright field (A, C) and dark field (B, D) microscopy. The section in (A, B) is approximately 42 µm more cranial than the section in (C, D). **(A, B)** Strong signal was localized in the somites, and in the mesenchyme surrounding the anterior cardinal vein (acv), bronchi (b), and foregut (fg). **(C, D)** Signal was localized in the somites, in the mesenchyme surrounding the anterior cardinal vein (acv), dorsal aorta (da), and foregut (fg), as well as the umbilical vein (uv). In both sections, a signal above background was also detected in the septum primum (sp), and the inner layer of the atrial wall. Red blood cells inside the atrium show non-specific signal. neural tube (nt); left atrium (la); atrioventricular canal (avc); bulbus cordis (bc); ventricle (v). Bar = 290 µm for A and B; 300 µm for C and D.

endocardium was positively labeled as reported previously (Kume et al., 1998).

Day 13 p.c. Embryos

As development proceeds, the spina vestibuli fuses with the inferior atrioventricular cushion, which in turn fuses with the superior atrioventricular cushion. This leads to the closure of the ostium primum by day 12 p.c. (Webb et al., 1998). At day 13 p.c., aortic and pulmonary trunks are separated and aortic and pulmonary valves are formed (Webb et al., 1998). *Mf1* expression was observed in the ventral part of the septum primum (Fig. 5), which is continuous with the atrioventricular cushion and forms the atrial septum (as). Hybridization signal was also detected in the venous valve (vv), the mitral valve (mv), the tricuspid valve (tv), and in the trabeculated region of the ventricular wall (Fig. 5). The density of silver granules is highest in the endocardium and appears to decrease in the mesenchymal cells that have migrated into the cushion tissue,

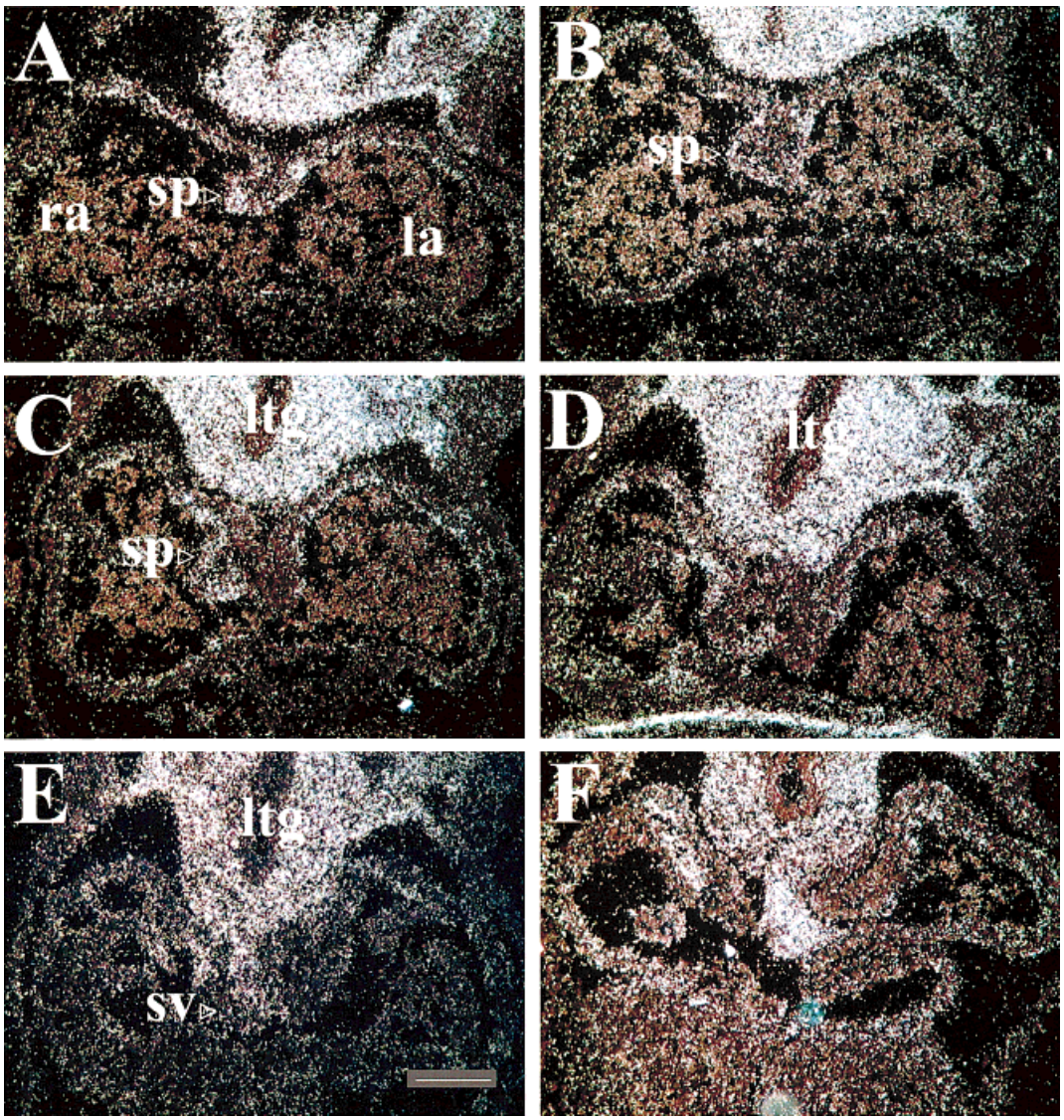

Fig. 3. Detection of *Mf1* transcripts in the hearts of day 11 p.c. embryos by in situ hybridization. Antisense (A-F) *Mf1* riboprobes were hybridized to serial cross-sections (7 μm) of a day 11 p.c. embryo and analyzed using dark field microscopy. Shown here are every fourth section from cranial (A) to caudal (F). A strong signal was detected in the mesenchyme surrounding the laryngeo-tracheal groove (ltg). A weak but significant signal was also detected in the inner wall of the atrium (A, C), in the septum primum (sp), and in the spina vestibuli (sv). right atrium (ra); left atrium (la). Bar = 115 μm. In D, the line of high background at the bottom of the picture is due to an artifact.

valve leaflet, and septum (Fig. 5). However, there was no significant level of signal detected in the interventricular septum (ivs) (Fig. 5). Expression was also noted in the aortic valve (av) and the pulmonary valve (pv) (Fig. 6).

Day 15 p.c. Embryos

By day 15 p.c., the interventricular septum is closed and septation is complete. In general, the intensity of *Mf1* signal was decreased at this stage compared to earlier stages. However, expression was still detected in the atrial septum (as), the venous valve (vv), the mitral valve (mv), the tricuspid valve (tv), the aortic valve (av) and the pulmonary valve (pv) (Fig. 7).

Clinical Patients With ARA and Congenital Heart Defects

Recently, we identified a proband with ARA and an atrial septal defect (Fig. 8, family 5, individual III-4). His mother (II-2) was diagnosed with ARA and reported to have hearing loss. The maternal grandfather (I-1) was found to have glaucoma and sensorineural hearing loss. All other members of the family 5 pedigree (Fig. 8) were examined ophthalmoscopically and found to be normal. Screening of *FKHL7* in this family revealed a 1

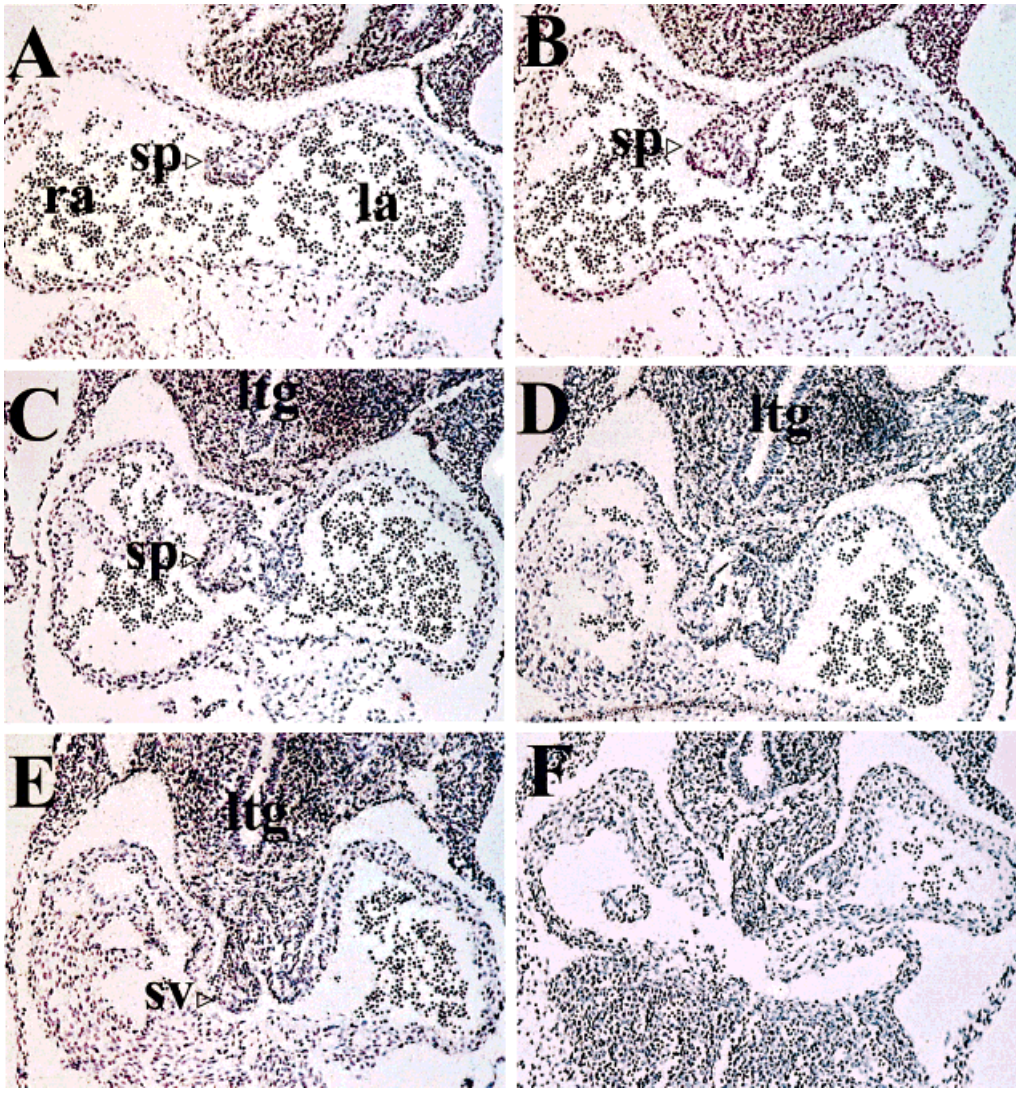

Fig. 4. Detection of *Mf1* transcripts in the hearts of day 11 p.c. embryos by in situ hybridization. Bright field micrographs corresponding to the dark field micrographs shown in Figure 3.

bp deletion (210/\G) upstream of the forkhead/winged helix DNA-binding domain. This deletion causes a frameshift in the translation of *FKHL7* that results in the alteration of 7 amino acids followed by premature termination, yielding a truncated molecule of 76 amino acids (full length *FKHL7* is 533 amnio acids). This truncated transcript lacks the DNA-binding motif and is likely to be nonfunctional. This change was inherited in both affected individuals and was not found among 128 normal Caucasian individuals.

Identification of this mutation prompted us to review the clinical histories of four previously published families in which we had identified mutations in *FKHL7* (Nishimura et al., 1998). No clinical manifestation of congenital heart defects could be found in families 1, 2, or 3. In family 4 (Fig. 8) three of the affected individuals (II-1, III-2, and III-4) with the eye phenotype were also reported to have mitral valve anomaly. This family was previously found to have a Phe112Ser missense mutation within the forkhead/winged helix domain of *FKHL7*. An ARMS (amplification-refractory mutation system) assay was developed for this mutation and was used to confirm the inheritance of this mutation in all affected individuals in the family. Individuals found to harbor the Phe112Ser mutation within this family were evaluated by echocardiography at the University of Iowa Hospitals and Clinics. Individual II-1 had mild tricuspid and mild mitral valve insufficiency. This patient also had an aortic valve prosthesis that was thought to be secondary to a primary congenital abnormality. Individual III-2 and III-4 had mitral valve insufficiency. All other individuals had normal echocardiograms. We

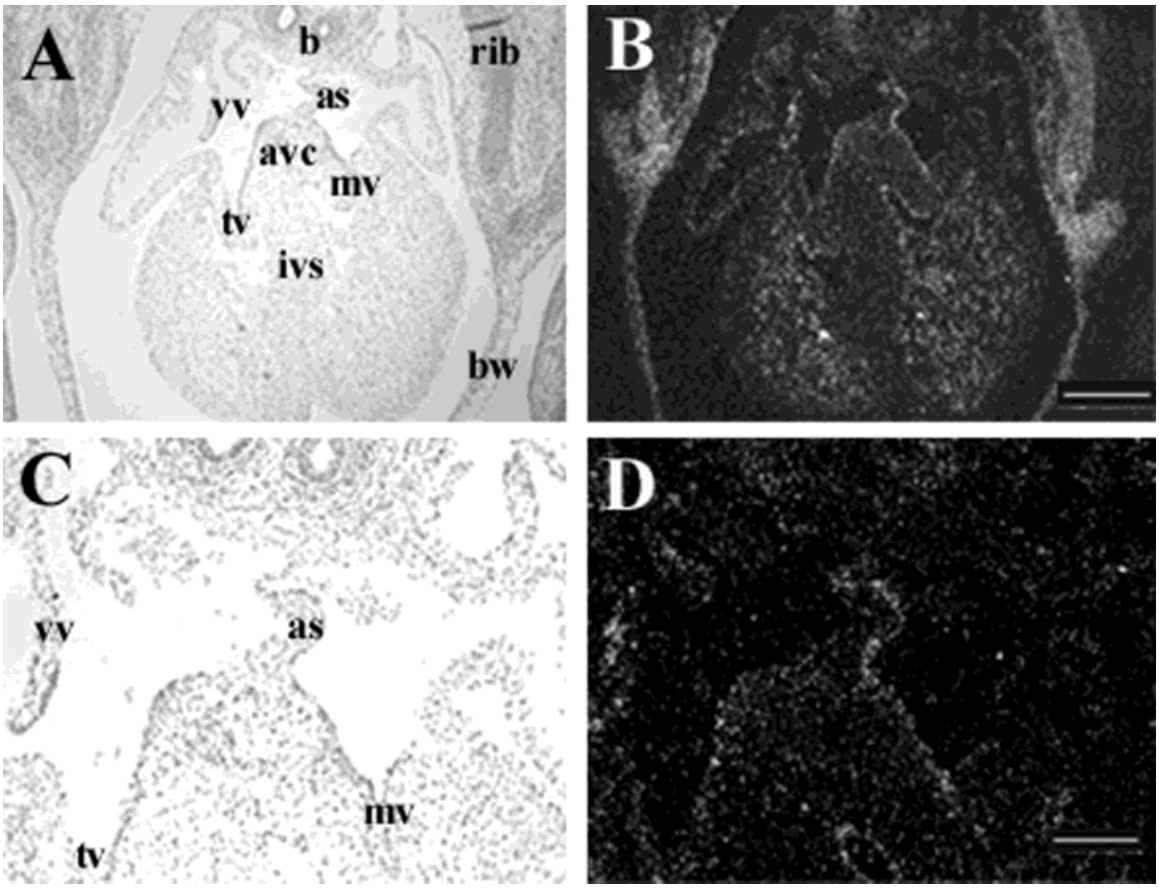

Fig. 5. Detection of *Mf1* transcripts in day 13 p.c. embryos by in situ hybridization. Antisense *Mf1* riboprobe was hybridized to embryo cross-sections and analyzed using bright field (A, C) and dark field (B, D) microscopy. **(A, B)** Signal was observed in the pre-chondrogenic mesenchyme of the rib, in the mesenchyme surrounding the bronchi (b), and in the inner cell layer of the thoracic body wall (bw). Within the heart, a positive signal was detected in the ventral component of atrial septum (as), in the atrioventricular cushion tissue (avc), in the mitral valve (mv), in the tricuspid valve (tv), in the venous valve (vv) and in the trabeculated region of the ventricular wall. However, no signal was detectable in the interventricular septum (ivs). Bar = 290 μm. **(C, D)** As seen at higher magnification, the strongest signal was associated with the cell layers covering the septae and valves, including the atrial septum (as), mitral valve (mv), tricuspid valve (tv), and venous valve (vv). The intensity of signal decreased as the cell layers reached inside of these structures. Bar, 115 μm.

have screened additional 128 controls by ARMS using a DNA pooling strategy and have not detected this change. Of the three mutations originally reported that were located within the forkhead/winged helix domain, this change is the only one that has been conserved across all the known gene family members as well as in the *Drosophila* orthologue (Nishimura et al., 1998).

DISCUSSION

Forkhead/winged helix transcription factors play critical roles during early embryonic and tissue-specific gene expression. In this report, we present new evidence that *Mf1*, the murine homologue of *FKHL7/FREAC3*, contributes to mouse heart valve formation and atrial septation. Using in situ hybridization analysis, we have shown that *Mf1* is expressed in the atrial septum, the venous, aortic and pulmonary valves, as well as the mitral and tricuspid valves but not in the interventricular septum. The temporal expression of the *Mf1* gene in the heart appears to be coincident with the time period critical for the formation of cardiac valves and septae, suggesting that the Mf1 protein may play important roles in the differentiation of valvular and septal tissues. Consistently, most of these tissues contain mesenchyme-derived cells, in which forkhead/winged helix family proteins are expressed.

Current thinking is that most congenital anterior segment anomalies are the result of aberrant migration or terminal differentiation of neural crest cells (Kupfer and Kaiser-Kupfer, 1978; Kaiser-Kupfer, 1989; Kappetein et al., 1991). Consistent with this assessment are the eye abnormalities observed in heterozygous and homozygous null *Mf1* mice. These mutant mice are presumed to result from a primary defect in the neural crest-derived periocular cells that express *Mf1* (Kume et al., 1998; Mears et al., 1998). A number of case reports have implied an association between defects of the anterior segment of the eye and congenital heart

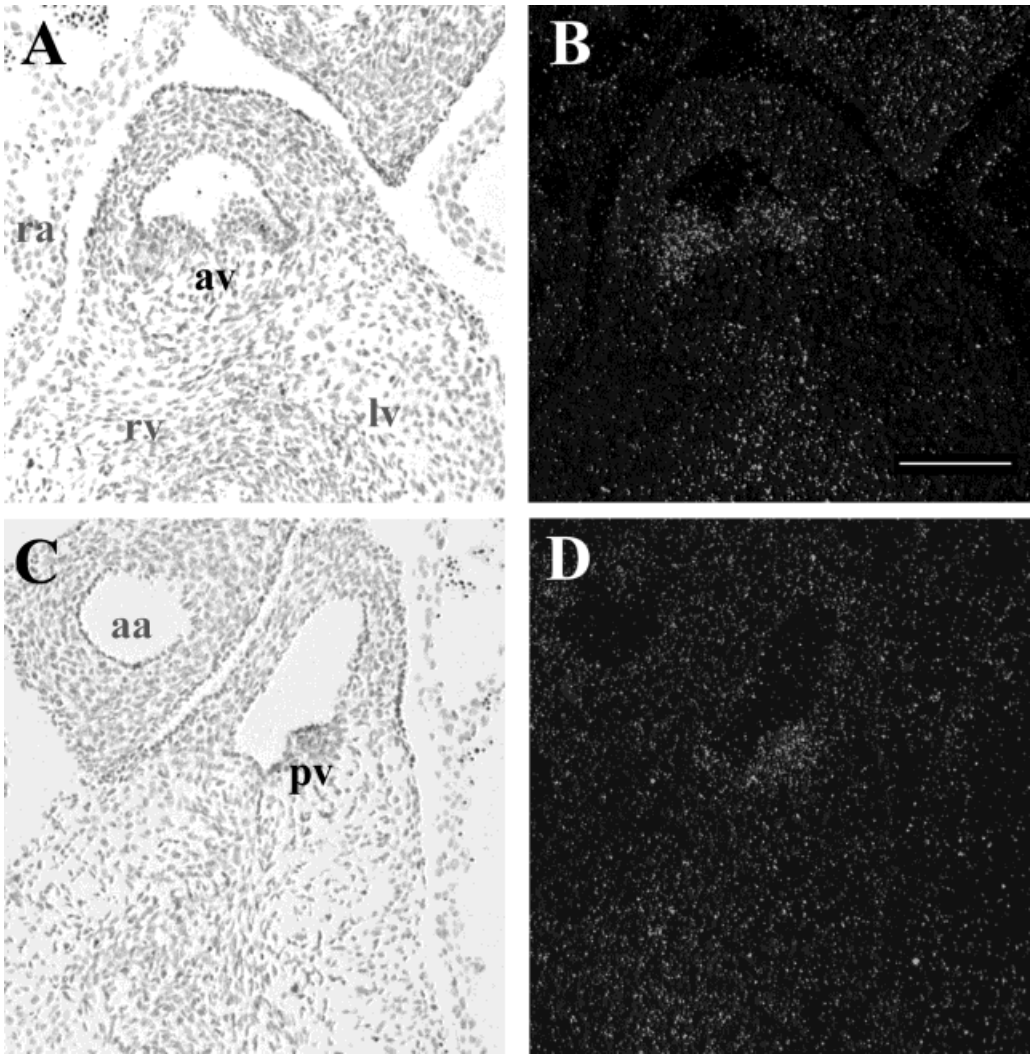

Fig. 6. Detection of *Mf1* transcripts in developing aortic and pulmonary valves of the day 13 p.c. embryonic heart. Antisense *Mf1* riboprobe was hybridized to embryonic cross-sections and analyzed using bright field (A, C) and dark field (B, D) microscopy. **(A, B)** *Mf1* expression was observed in the developing aortic valves (av) and in the tissue between right and left ventricles (rv and lv, respectively). right atrium (ra). **(C, D)** *Mf1* expression was observed in the developing pulmonary valve (pv). aa, ascending aorta. Bar = 115 μm

disease (Tsai and Grajewski, 1994; Cunningham et al., 1998; Law et al., 1998; Mammi et al., 1998). During heart development, the cardiac neural crest is essential for septation of the cardiac outflow tract and for aortic arch artery development (Creazzo et al., 1998; Waldo et al., 1998). Malformations in these regions result in coarctation of the aorta which is frequently associated with a bicuspid aortic valve (Kappetein et al., 1991), both of which have been observed in some patients with eye anterior segment defects (Law et al., 1998; Mammi et al., 1998). Based on the results of our study, the expression pattern of *Mf1* in developing mouse hearts suggest that *Mf1* contributes not only to structures derived from cardiac neural crest cells (that is, the aortic and pulmonary valves of the outflow tract, but interestingly, not the interventricular septum), as well as structures derived from a range of embryonic origins. These include the endocardial cushion cells that form the mitral and tricuspid valves, and the extracardiac mesenchyme (spina vestibuli) from the developing pulmonary vein which gives rise to the future atrial septum. The expression of *Mf1* in the developing spina vestibuli and mitral valve may account for the cardiac anomalies observed in members of two families with *FKHL7* mutations, notably atrial septal defects and mitral valve anomalies.

The occasional occurrence of Axenfeld-Rieger *FKHL7* mutation-bearing individuals with clinical cardiac phenotypes, and the diversity of the cardiac defects observed in our kindreds suggest that whereas disease penetrance in the eye is high, it is incomplete with variable expressivity in the heart. This implies that *FKHL7* mutations confer disease susceptibility in the heart that requires environmental factors or additional

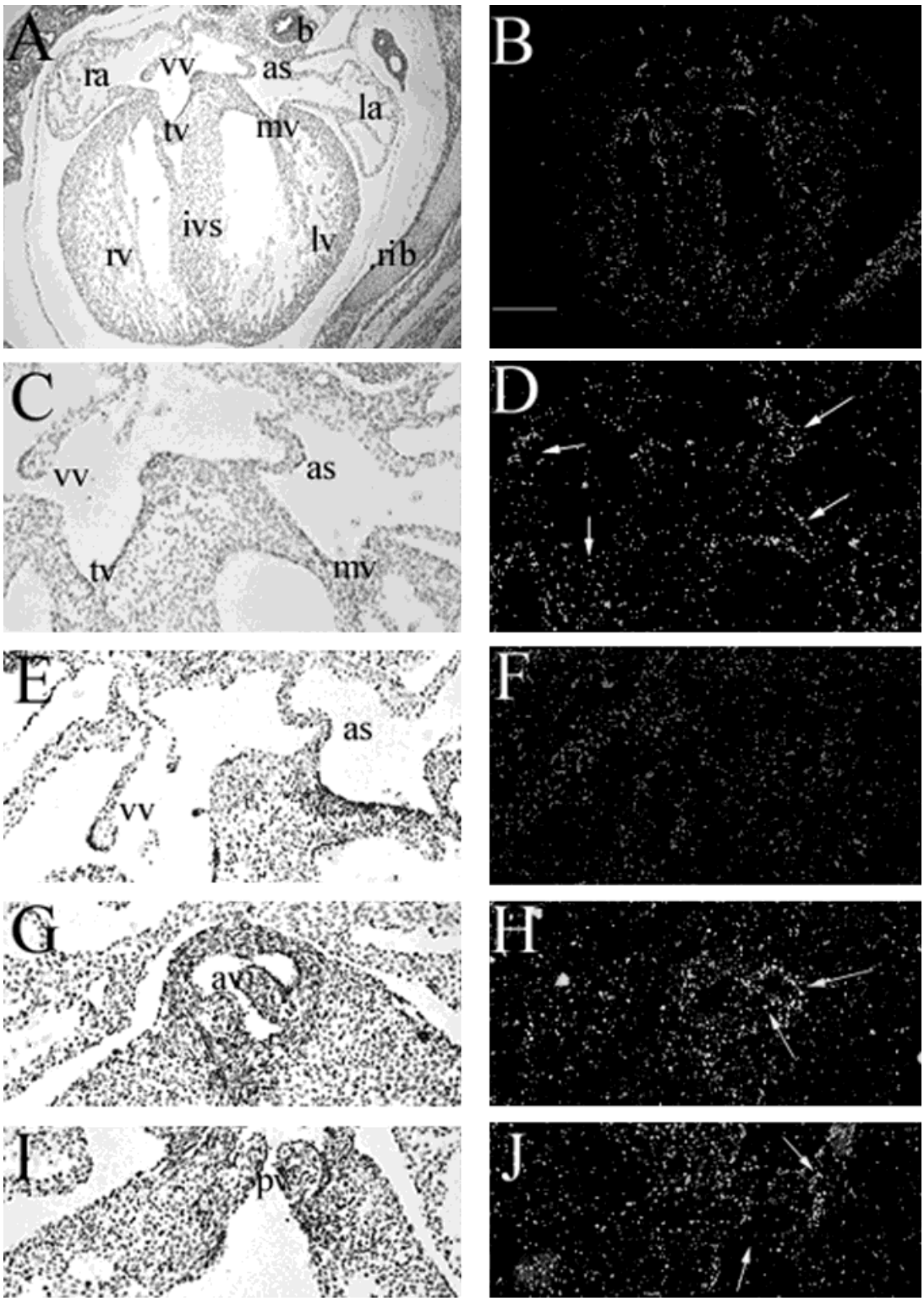

Fig. 7. Detection of *Mf1* transcripts in the day 15 p.c. embryonic heart. Antisense *Mf1* riboprobe was hybridized to embryo cross-sections and analyzed using bright field (A, C, G, I) and dark field (B, D, H, J) microscopy. Sense *Mf1* probe was used as a control (E, F). **(A, B)** low magnification and **(C–J)** high magnification: The signal intensity in the heart of day 15 p.c. embryo was much weaker than that of day 13 p.c. embryo. However, *Mf1* expression was still observed in the atrial septum (as), mitral valve (mv), tricuspid valve (tv) and venous valve (vv) (indicated by white arrows in D), as well as in the trabeculated regions of the ventricles. No significant signal was detected in these structures with the sense probe (E, F). A signal above background was also observed in aortic valve (av), pulmonary valve (pv), and tissues near these valves (indicated by white arrows in H, J)). bronchus (b); right atrium (ra); left atrium (la); right ventricle (rv); left ventricle (lv); interventricular septum (ivs). Bar = 290 μm for A and B; and 115 μm for C–J.

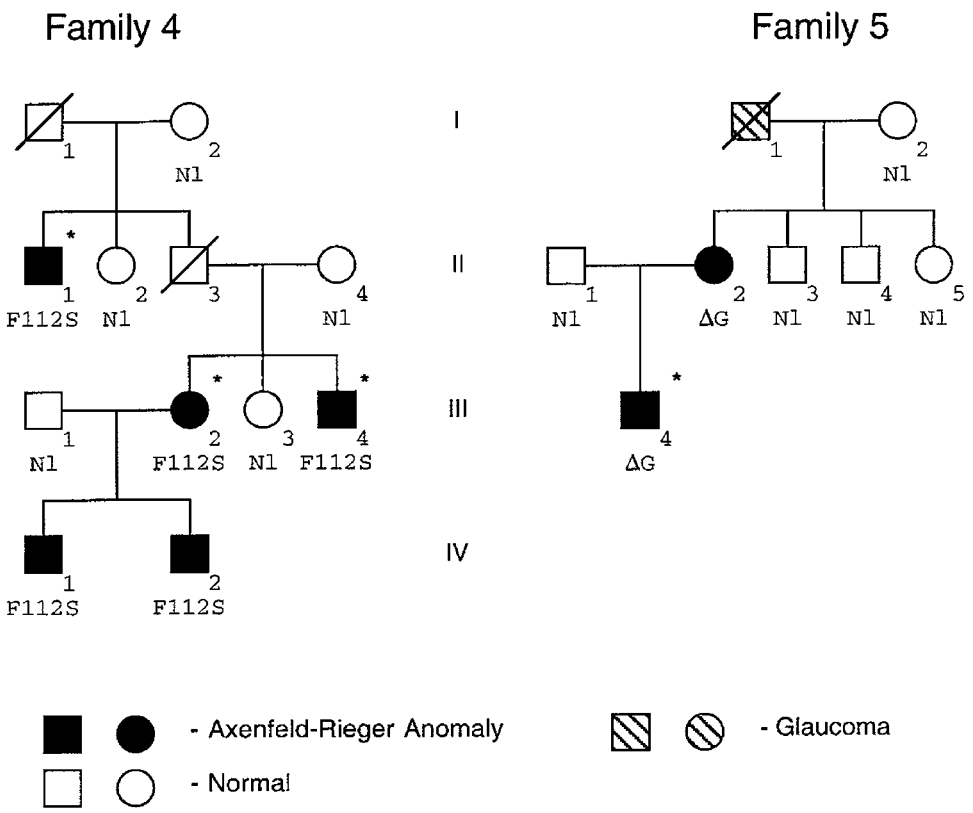

Fig. 8. The pedigree structure of families 4 and 5 with anterior segment defects and mutations in *FKHL7*. The numbering of these families is in accordance with our previously published pedigrees (Nishimura et al., 1998). Family 4 has a Phe112Ser missense mutation in the *FKHL7* gene. The following individuals of family 4 were not available for study (I-1, I-2, and II-3). Asterisk (*) refers to mitral valve defects. Family 5 has a one base pair deletion (210^G) upstream of the DNA-binding domain of the *FKHL7* gene. This results in a truncated protein lacking the DNA-binding domain. In family 5, individual I-1 was not available for study, and the phenotype of individual II-4 was determined from medical records. The glaucoma subtype of individual I-1 is unknown. Asterisk (*) refers to atrial septal defect.

genetic loci to manifest the disease. Studies of several forms of congenital heart disease such as atrioventricular canal defects (AVCD) associated with chromosome 1 (Sheffield et al., 1997), trisomy 21 (Down syndrome) (Rowe and Uchida, 1961, Ferencz et al., 1989; Marino et al., 1990), trisomy 16 in mice (Cox et al., 1984) and the secundum type of atrial septal defect on chromosome 5p (Schott et al., 1998) have also been shown to be associated with mutations that are not fully penetrant. In the case of *FKHL7* mutations, expressivity in the heart may reflect the influence of a modifying gene or environmental factors. In this regard, the identification and characterization of upstream genes that regulate *FKHL7* gene expression are needed. For example, there may be functional compensation by other forkhead/winged helix genes. *FREAC5/FKHL9* (9p11–q11), another member of this family of transcription factors is expressed in human fetal and adult heart tissue as assessed by Northern blotting (Pierrou et al., 1994), suggesting that this gene might be a good candidate for a modifying gene. *Mfh1*, another related forkhead/winged helix family member shows a striking overlap in expression compared to *Mf1*, as well as similar DNA binding domain homology (Miura et al., 1993; Kaestner et al., 1996; Iida et al., 1997; Winnier et al., 1997; Hiemisch et al., 1998a; Kume et al., 1998). Another potential candidate for a modifying gene in the heart is *Mfh3* which is also expressed in adult mouse heart as assessed by Northern blotting, although its temporal expression pattern in the developing embryo is somewhat delayed as compared to *Mf1* (Kaestner et al., 1993). Thus, although there is no reported histology of heart defects in the heterozygous *ch* or *Mf1* mice to support or refute the modifying gene theory, the possibility of cross-breeding the commercially available *ch* mice onto different genetic backgrounds to test this hypothesis would be an interesting experiment.

The downstream targets of *Mf1* in the heart have not yet been elucidated. However, prechondrogenic mesenchyme cells derived from null mutant *Mf1* mice have a reduced ability to respond to the growth factors BMP-2 and TGFβ-1 and subsequently exhibit reduced chondrogenesis (Kume et al., 1998). This suggests a critical role for *Mf1* in the differentiation of these cells into cartilage. Similarly, the hierarchy of transcription factors implicated in notochord formation suggests that *Brachyury* (*T*) may act downstream of the winged helix protein *HNF-3*β in the mouse (Weinstein et al., 1994). Addi-

tional members of the winged helix family of proteins have been associated with the downstream signaling pathway of the growth factor activin in *Xenopus* and with DAF-7 regulation of development and metabolism in *C. elegans* (Chen et al., 1997; Ogg et al., 1997).

Existing reports of families with anterior segment defects mapping to chromosome 6p25 which lack a mutation within the *FKHL7* coding region has led to the suggestion that another gene near the *FKHL7* gene also results in anterior segment defects (Mears et al., 1998). We view this suggestion as controversial and not substantially supported at this time, although within the realm of possibility. We are currently identifying genes within the duplication/deletion region of 6p25 to screen selected genes within this region for mutations in anterior segment dysgenesis/glaucoma patients.

EXPERIMENTAL PROCEDURES

In Situ Hybridization

NIH Swiss mouse embryos and dissected hearts were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, dehydrated, cleared in xylene, and embedded in Paraplast Plus (Oxford). Serial 7 μ sections were cut and mounted onto Superfrost Plus slides (Fisher) and were hybridized with ^{35}S-labeled sense and antisense *Mf1* riboprobes generated from an *Mf1* cDNA-containing plasmid corresponding to the 3′ untranslated region (I.M.A.G.E. Consortium Clone 864300, Research Genetics) using T3 and T7 RNA polymerases. Hybridization with labeled sense RNA riboprobes served as controls for non-specific hybridization, and in all cases, no specific hybridization was observed. In situ hybridization was carried out as described by Angerer (Angerer et al., 1987). Briefly, tissue sections mounted on slides were hybridized overnight at 50°C in 50% formamide, 1X STE (0.3 M NaCl, 20 mM Tris pH 8.0, 1 mM EDTA), 80 μg/ml denatured salmon sperm DNA, 1X Denhardt's solution, 10 % dextran sulfate, 500 μg/ml yeast tRNA, and 0.1 M dithiothreitol (DTT). Following hybridization, slides were washed twice in 5X SSC-0.1 M DTT at 50°C for 30 min each, and once in 2X SSC-50 % formamide at 60°C for 30 min. After RNase treatment, slides were further washed in 2X SSC at 37°C, 0.1X SSC at 50°C, and 0.1X SSC at room temperature for 15 min each. After dehydration, slides were dipped in NT2-B photographic emulsion (Kodak) and exposed for 1–2 weeks at 4°C. Slides were developed, counterstained with hematoxylin and observed with bright field and dark field microscopy. The images were collected by a digital microscope camera (Polaroid) directly attached to a microscope and processed using Adobe photoshop.

Patients and Families

All of the patients in this study were from the U.S. Whole blood was drawn from probands and their family members following signed informed consent. Genomic DNA was prepared from whole blood as previously described (Stone et al., 1997). Patients examined ophthalmologically had either Axenfeld anomaly (AA) or Rieger anomaly (RA). AA was defined as posterior embryotoxon of the cornea with iris strands between the iris and trabecular meshwork. RA had the same findings as AA with the addition of iris hypoplasia.

Mutation Detection and Confirmation

The mutation in the proband of family 4 was detected by screening the 5′ portion of the coding sequence through the forkhead domain by sequencing using protocols that have been previously described (Nishimura et al., 1998). Confirmation of this mutation was accomplished by amplification-refractory mutation system (ARMS) analysis (Newton et al., 1989).

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