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Induction of avian cardiac myogenesis by anterior endoderm

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SUMMARY

An experimental system was devised to study the mechanisms by which cells become committed to the cardiac myocyte lineage during avian development. Chick tissues from outside the fate map of the heart (in the posterior primitive streak {PPS} of a Hamburger & Hamilton stage 4 embryo) were combined with potential inducing tissues from quail embryos and cultured in vitro. Species-specific RT-PCR was employed to detect the appearance of the cardiac muscle markers chick Nkx-2.5 (cNkx-2.5), cardiac troponin C and ventricular myosin heavy chain in the chick responder tissues. Using this procedure, we found that stage 4-5 anterior lateral (AL) endoderm and anterior central (AC) mesendoderm, but not AL mesoderm or posterior lateral mesendoderm, induced cells of the PPS to differentiate as cardiac myocytes. Induction of cardiogenesis was accompanied by a marked decrease in the expression of ρ-globin, implying that PPS cells were being induced by anterior endoderm to become cardiac myocytes instead of blood-forming tissue. These results suggest that anterior endoderm contains signaling molecules that can induce cardiac myocyte specification of early primitive streak cells.

One of the cardiac muscle markers induced by anterior endoderm, cNkx-2.5, is here described for the first time. cNkx-2.5 is a chick homeobox-containing gene that shares extensive sequence similarity with the *Drosophila* gene *tinman*, which is required for *Drosophila* heart formation. The mesodermal component of cNkx-2.5 expression from stage 5 onward, as determined by in situ hybridization, is strikingly in accord with the fate map of the avian heart. By the time the myocardium and endocardium form distinct layers, cNkx-2.5 is found only in the myocardium. cNkx-2.5 thus appears to be the earliest described marker of avian mesoderm fated to give rise to cardiac muscle.

Key words: heart, endoderm, cNkx-2.5, chick, myogenesis

INTRODUCTION

For the better part of a century, the vertebrate heart has been used as a model system for studying cell and tissue specification in the developing embryo. These studies have primarily utilized avian and amphibian species. In the early gastrula of the chick {stage 3 (Hamburger and Hamilton, 1951)}, myocardial precursor cells are located in the mid-primitive streak, from which they enter the mesoderm and spread anterolaterally (Rosenquist and DeHaan, 1966; Garcia-Martinez and Schoenwolf, 1993). By stage 6 (early neurula), the first precursor myocardial cells are approaching the region of the anterior intestinal portal at the ventral midline (Rosenquist and DeHaan, 1966) and the first reported markers of terminal myocardial differentiation are detected in this area at stage 7 (Bisaha and Bader, 1991; Han et al., 1992). In amphibians, the heart primordia have an analogous fate map: they are located in the deep mesoderm at the lateral blastopore lip of the early gastrula, from which they migrate anterolaterally and then ventrally to differentiate in the anterior ventral midline at the tailbud stage (Wilens, 1955).

The timing of cardiac myocyte specification has been investigated in both amphibians and birds. In several urodele species, explants of cardiac primordia taken from neurula and later stages will differentiate into beating cardiac muscle in culture (Jacobson, 1961) while, in the anuran *Xenopus laevis*, this degree of specification is reached significantly earlier, by mid-gastrula stages (Sater and Jacobson, 1989). In birds, individual cells taken from stage 3 (early gastrula) embryos will not differentiate as cardiac muscle (Montgomery et al., 1994), whereas individual cells or groups of mesodermal cells from the cardiogenic regions of stage 4-5 (mid-late gastrula) and older embryos will differentiate into cardiac myocytes (Gonzalez-Sanchez and Bader, 1990; Antin et al., 1994; Gannon and Bader, 1995), suggesting that cardiac myocyte specification in birds, as in *Xenopus*, occurs during gastrulation.

The mechanisms whereby embryonic cells come to adopt a cardiac fate are not well understood. In both amphibians and birds, the presumptive heart tissue (in the mesoderm) lies in close contact with endoderm from the time of gastrulation until terminal differentiation. Work from a number of groups has demonstrated that inductive interactions between endoderm and the overlying mesoderm play a role in promoting the differentiation of cardiac tissues. In urodeles, Jacobson and colleagues have shown that inclusion of anterior endoderm increases the percentage of presumptive heart explants from early neurula embryos that differentiate into beating heart

muscle (Jacobson, 1960; Jacobson, 1961; Jacobson and Duncan, 1968). Similarly, deep endoderm promotes the differentiation of the overlying heart primordia at early gastrula stage in *Xenopus laevis* (Nascone and Mercola, 1995). In the chick, Orts-Llorca and colleagues have shown that removal of the endoderm underlying the precardiac region from stage 5 and older embryos produces defects in cardiogenesis (Orts-Llorca, 1963). Sugi and Lough have reported that stage 6 anterior lateral (AL) mesoderm will not differentiate into cardiac muscle when cultured with overlying ectoderm, but will do so when co-cultured with underlying AL endoderm (Sugi and Lough, 1994). However, in all of these studies the myocardial-inducing capacity of endoderm was assayed with tissues that were normally fated to give rise to heart. Thus, it is unclear in these cases whether the inducing activity of the endoderm is instructive (i.e. influencing lineage decisions in the responding tissue) or permissive (i.e. promoting differentiation of already committed precardiac myocytes).

A few studies in amphibians suggest that endodermal tissues may in fact participate in cardiac myocyte lineage decisions. Beating heart can be produced in the urodele *Amblystoma punctatum* by transplanting non-cardiogenic regions of the gastrula embryo into a region adjacent to the archenteron floor (i.e., pharyngeal endoderm, the region of the embryo where heart will terminally differentiate), suggesting that endoderm from this area may play a role in specification of heart tissues (Bacon, 1945). Moreover, Nascone and Mercola have reported that deep endoderm underlying the gastrula heart primordia can promote development of heart tissue from non-cardiogenic regions of the *Xenopus* embryo, but only when organizer tissue is also present (Nascone and Mercola, 1995).

Here we report a new system for studying avian myocardial specification. To distinguish between instructive and permissive inductive interactions, we have studied cardiac myogenesis in explants from the PPS, which is not normally fated to give rise to heart. Use of quail/chick chimeras and species-specific PCR has allowed us to assay for inducing activity in tissues that may themselves contain myocardial precursors. We have used this experimental approach to characterize cardiac muscle-inducing activity in the gastrula-stage chick embryo.

MATERIALS AND METHODS

Cloning, library screening and sequencing of *cNkx-2.5*

Degenerate primers A and B were directed at regions that are 100% conserved at the amino acid level in *Drosophila tinman* and mouse *Nkx-2.5* (see Fig. 1 for position of primers):

A: 5′-CC(AGCT) TT(CT) TCI GTI AA(AG) GA(CT) AT-3′
B: 5′-C(TG) (AG)TT (CT)TG (AG)AA CCA (AGT)AT (CT)TT-3′

cDNA was synthesized from embryonic day 4 chick and quail heart RNA, and PCR was performed as described below in the 'RT-PCR' section, except that the following amplification conditions were used: (1) initial denaturation at 99°C for 5 minutes; (2) add 3 Units Taq polymerase in 'hot start'; (3) 5 cycles of: denaturation at 97°C for 15 seconds, anneal at 37°C for 2 minutes, slow ramp (1°C/3 seconds) to 72°C, and extension at 72°C for 2 minutes; (4) 35 cycles of: 97°C for 15 seconds, 38°C for 2 minutes, 72°C for 2 minutes; (5) extension at 72°C for 5 minutes. Formamide (5%) was included in the reaction mixture.

PCR products of the expected size were gel purified and ligated into pT7Blue (Novagen). Three chick and three quail clones were partially sequenced from the double-stranded plasmids using Sequenase (USB) and standard dideoxy methodology. The sequences of all three chick inserts and two of the quail inserts showed significant homology with the mouse and *Xenopus* Nkx-2.5 genes. The chick PCR insert was labelled with [^{32}P]-α-dCTP using random priming and the labelled probe was used to screen approximately 600,000 plaques of a stage 12-15 chick embryo λZAPII cDNA library, kindly provided by M. Angela Nieto (Nieto et al., 1994). Three positive clones were identified and excised using helper phage as Bluescript II SK– plasmids (Stratagene). Agarose gel electrophoresis revealed that the clones had inserts of 1.5, 1.6 and 3 kb. Partial sequencing of each clone (as above) revealed that the 1.6 kb clone contained a poly(A) tail and an open reading frame with significant homology to the 5′ end of the mouse Nkx-2.5 gene, and this clone was sequenced in its entirety in both directions using standard dideoxy methodology. Alignment of the predicted cNkx-2.5 protein sequence with the *Xenopus* and mouse homologues was performed using the Clustal method with MegAlign software (DNASTAR).

Whole-mount in situ hybridization and sectioning

The procedure was performed essentially as described in Wilkinson (1993) with the indicated modifications. Digoxigenin-labelled RNA probes were synthesized from full-length cNkx-2.5 in Bluescript II SK– by cutting with *Hin*dIII and transcribing with T3 RNA polymerase (antisense), or cutting with *Xba*I and transcribing with T7 RNA polymerase (sense). Embryos were treated with 10 μg/ml Protease K (Sigma) for 15 minutes (stage 7 and younger) or 25 minutes (stage 8 and older). Probe concentration in the hybridization mix was approximately 0.2 μg/ml. The alkaline phosphatase reaction was developed for 2 to 24 hours. No signal was ever detected with the sense probe.

Embryos to be sectioned were infiltrated overnight in 20% sucrose in PBS at 4°C and then embedded in Peel-A-Way plastic molds (VWR) in 7.5% gelatin (Sigma)/15% sucrose/1× PBS. After the gelatin had set, blocks were trimmed and then frozen on cryostat chucks submerged in liquid nitrogen (the blocks themselves were not submerged). 20 μm cryostat sections (Microm) were taken up on uncoated glass slides and mounted with Gelvatol (36 ml 0.02 M NaH_2PO_4, 14 ml 0.02 M KH_2PO_4, 0.327 g NaCl, 0.024 g NaN_3, 10 g polyvinyl alcohol {30-70, low temperature soluble, Sigma}, 20 ml glycerol, pH=7.2). Whole-mount embryos were photographed through a Zeiss dissecting microscope and sections were photographed using a Zeiss Axiophot microscope with Nomarski optics; in both instances, Kodak Ektachrome ASA 64 Tungsten film was used.

Embryonic cultures

Fertile chick (Spafas) and quail eggs (Trusslow Farms, Chestertown, MD) were incubated at 38.5ºC in a humidified incubator (Petersime) and staged according to Hamburger and Hamilton (1951), as modified by Schoenwolf et al. (1992). Embryos with a portion of the attached vitelline membrane were removed into a dish containing Tyrode's saline (TS; NaCl 8 g/l, KCl 0.2 g/l, $CaCl_2$ 0.2 g/l, NaH2PO_4 50 mg/ml, $MgCl_2$ 0.2 g/l, Glucose 1 g/l). Embryos were dissected away from the vitelline membrane with a tungsten needle and transferred to dissecting dishes (60 mm Petri dishes containing a layer of hardened 1% agar) in TS. Embryos were pinned, ventral side up, by pushing the edges of the area opaqua into the agar with tungsten needles. The appropriate areas of each embryo were dissected with fine tungsten needles and stored in a drop of Chick Embryo Medium (CEM: DMEM α-modification (Gibco), with 10% fetal bovine serum, 5% chick embryo extract, 1% L-Glutamine and 1% Pen/Strep) on ice. The PPS explants were taken from the posteriormost segment of the primitive streak of stage 3c-4 chick embryos (c_c in Inagaki et al., 1993), included the full thickness of the streak, and measured approximately 175 μm long × 125 μm wide.

Explants were cultured on floating filter rafts, as described by

Holtzer and colleagues (Lash et al., 1957). Wells of a 24-well plate (Falcon) were filled with 1 ml of CEM, and a Nucleopore filter (0.4 μm, Costar) was placed on top of the medium. Explants were deposited onto the filter in a drop of CEM and positioned as the CEM seeped through the membrane. In order to achieve adequate contact between tissues, it was necessary to position the quail tissues with their ventral side in contact with the filter. That way, as the medium seeped through the filter, the tissue 'unrolled' into a flat sheet, onto which primitive streak explants were positioned. In this manner, it was assured that the responding tissue (chick PPS) was in contact with that face of the quail-inducing tissue which is normally facing the interior of the embryo. Cultures were incubated for 48 hours in a humidified incubator at 37°C and 5% CO_2.

RT-PCR

Cultures were harvested in 200 μl of Solution D (25 g guanidinium thiocyanate, 29.3 ml water, 1.76 ml 0.75 M sodium citrate pH=7, 2.64 ml 10% Sarkosyl, 38 μl β-mercaptoethanol), extracted with water-saturated phenol and chloroform and precipitated with ethanol, with 20 μg glycogen (Boehringer-Mannheim) added as a carrier. Samples were treated with 2 Units of RNAse-free DNAse (Boehringer-Mannheim) for 2 hours at 37°C in 50 μl of DNAse buffer (40 mM Tris pH=8.0, 10 mM NaCl, 6 mM $MgCl_2$) containing 2 Units of ribonuclease inhibitor, followed by phenol/chloroform extraction and ethanol precipitation. Reverse transcription was carried out for 1 hour at 42°C in 30 μl of RT buffer (as supplied by GIBCO-BRL), supplemented with 0.5 mM of each dNTP (Boehringer-Mannheim), 3.3 mM dithiothreitol, 2 Units of ribonuclease inhibitor, 200 units of Maloney murine leukemia virus reverse transcriptase (GIBCO-BRL) and 200 ng of a random hexamer for priming.

Polymerase chain reaction (PCR) was carried out in a volume of 50 μl, using PCR buffer (Boehringer-Mannheim), 1 Unit of Taq polymerase (Boehringer-Mannheim), 0.2 mM each dNTP (Boehringer-Mannheim), 0.1 μl [^{32}P]-α-dCTP (NEN), 500 ng of each primer, and 1 μl of template (from a 30 μl RT reaction). For PCR of cNkx-2.5, MyoD and myogenin, 5% deionized formamide was added. Thermal cycling was performed in a MJ-Research thermal cycler as follows: (1) initial denaturation at 93°C for 3 minutes, (2) cycling for the indicated number of cycles (see below) between 93°C for 30 seconds, the annealing temperature (see below) for 30 seconds and 72°C for 90 seconds, (3) final extension at 72°C for 5 minutes. Primers were selected to span restriction endonuclease sites that differed between chick and quail genes. The number of cycles was chosen so that amplification remained well within the linear range, as assessed by phosphorimager analysis (Molecular Dynamics). Consistent with the high degree of similarity between the chick and quail genomes (for cTnC and GAPDH, where both chick and quail sequences are known, the homologues are greater than 96% identical at the nucleotide level) we found that the primers that we utilized amplified both chick and quail homologues with similar efficiency.

Gene	Primers	Product length (bp)	Anneal temp. (°C)	Cycles
cNkx-2.5	5′-CCTTCCCCGGCCCCTACTAC-3′ 5′-CTGCTGCTTGAACCTTCTCT-3′	221	51	30
cTnC	5′-ATCTATAAGGCGGCGGTTGA-3′ 5′-CAGTGATCGTCTCTCCAGTT-3′	379	56	30
vMHC	5′-AAAGGTGGCAAGAAACAAT-3′ 5′-TGCTTTTGGTTATTCCTGAT-3′	457	52	29
ρ-globin	5′-GCGTCTGGAGCAAAGTCAAC-3′ 5′-TGGCAGGTCGGGGTGAAGTC-3′	343	56	21
GAPDH	5′-ACGCCATCACTATCTTCCAG-3′ 5′-CAGCCTTCACTACCCTCTTG-3′	578	56	23
MyoD	5′-CGTGAGCAGGAGGATGCATA-3′ 5′-GGGACATGTGGAGTTGTCTG-3′	280	55	30
Myogenin	5′-AGCCTCAACCAGCAGGAG-3′ 5′-TGCGCCAGCTCAGTTTTGGA-3′	284	55	30

5 μl of each PCR (except MyoD and myogenin) was digested for 3 hours in a total volume of 10 μl with 2 Units of a restriction enzyme (NEB) that cut the chick and quail gene products differentially. The following enzymes were utilized: GAPDH, *Sac*I + *Nsi*I (in Nsi buffer); cTnC, *Sac*I; cNkx-2.5, *Hin*fI; vMHC, *Taq*αI; ρ-globin, *Bst*EII. Following restriction enzyme digest, 2 μl of 6× loading buffer was added, and the samples were electrophoresed on a 6% polyacrylamide gel. Dried gels were either exposed overnight to X-ray film (Fuji), or imaged with a Molecular Dynamics phosphorimager. Phosphorimager data was quantified using ImageQuant software (Molecular Dynamics). References for the chick and quail sequences are: cNkx-2.5: this paper; cTnC: (Maisonpierre et al., 1987; Toyota et al., 1989); vMHC: (Bisaha and Bader, 1991); ρ-globin: (Dodgson et al., 1983); GAPDH: (Panabieres et al., 1984; Weiskirchen et al., 1993); MyoD: (Lin et al., 1989); myogenin: (Fujisawa-Sehara et al., 1990).

RESULTS

Cloning and expression pattern of chick Nkx-2.5

To clone the chick Nkx-2.5 (*cNkx-2.5*) gene, degenerate PCR primers that encode a conserved decapeptide at the 5′ end of the gene and a conserved region of the homeobox (Bodmer et al., 1990; Komuro and Izumo, 1993; Lints et al., 1993) were employed to amplify a 500 bp fragment from day 4 chick embryo heart cDNA. This PCR fragment was used as a probe to screen a stage 12-15 chick embryo cDNA library. A cDNA clone was isolated containing a 294 amino acid open reading frame, along with 5′ and 3′ untranslated regions of 78 and 573 bp, respectively (Fig. 1). An in frame stop codon is located 39 bp 5′ of the initial ATG, suggesting that the entire open reading frame has been cloned. The predicted cNkx-2.5 amino acid sequence contains an amino-terminal decapeptide conserved between vertebrate Nkx-2.5 genes and Drosophila *tinman*; a homeobox domain containing a tyrosine at position 123 which is characteristic of the NK class of homeobox genes; and a 15 amino acid stretch C-terminal to the homeobox which is conserved among members of the NK-2 class of homeodomain proteins (Lints et al., 1993). Comparison of the predicted amino acid sequence of cNkx-2.5 with the mouse (Komuro and Izumo, 1993; Lints et al., 1993) and *Xenopus* (Tonissen et al., 1994) Nkx-2.5 gene products demonstrates that the proteins are closely related (see Fig. 1, legend). The same degenerate primers were also used to amplify an approximately 500 bp fragment of the quail Nkx-2.5 gene from day 4 quail embryo heart cDNA, which was sequenced (data not shown) and used to identify a restriction site polymorphism that distinguishes it from the corresponding region of the chick gene (see below).

The expression pattern of *cNkx-2.5* was analyzed during chicken development by whole-mount in situ hybridization (Fig. 2). *cNkx-2.5* expression was first detected In stage 5 embryos, where it formed a crescent extending from the lateral plate at the level of Hensen's node to a point anterior to the tip of the head process (Fig. 2A). By stage 6, the *cNkx-2.5* signal was significantly stronger and the anterior margin of expression was localized at the anterior intestinal portal (AIP) (Fig. 2B). Sections from this stage embryo revealed that the lateral stripes of *cNkx-2.5* expression were mesodermal (Fig. 2G,H), whereas expression at the AIP (where the mesoderm has not yet reached) was evident in both ectoderm and

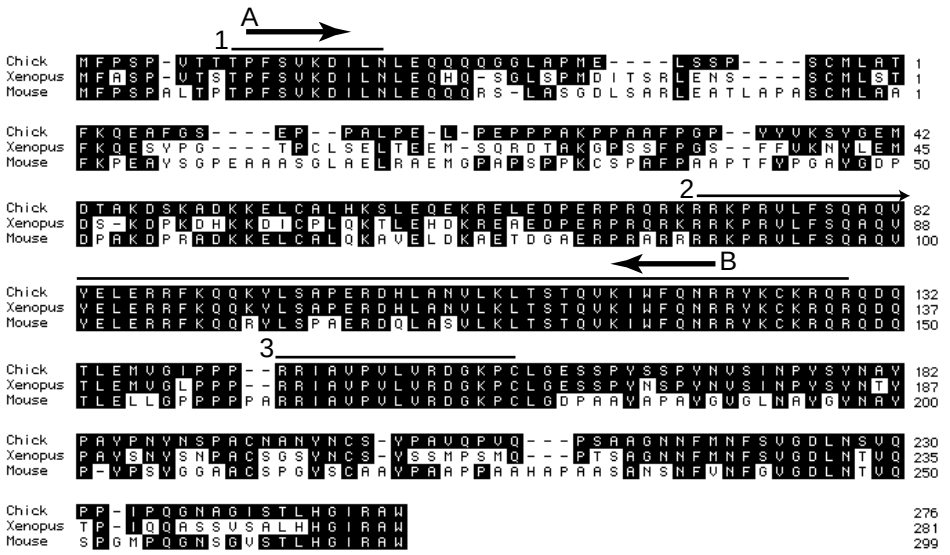

Fig. 1. Predicted amino acid sequence of cNkx-2.5, aligned with the *Xenopus* and mouse homologues. The numbered features overlying the sequence indicate: (1) decapeptide conserved between vertebrate Nkx-2.5 and *Drosophila* tinman proteins; (2) the homeobox; and (3) 'NK-2' domain conserved between vertebrate NK-2 class proteins and *Drosophila* NK-2. The forward (A) and reverse (B) arrows overlying the sequence indicate the positions of the degenerate PCR primers used in the initial amplification of cNkx-2.5 from 4 day embryonic chick heart cDNA. The chick and *Xenopus* proteins display 100% identity in the homeobox and 75% overall identity; the chick and mouse proteins display 92% identity in the homeobox and 67% identity overall.

endoderm (Fig. 2F,G). By stage 7, expression appeared in the ectoderm and endoderm directly overlying and underlying the lateral mesodermal expression stripes (Fig. 2I). By stage 8+ (Fig. 2C), *cNkx-2.5* expression was found in the forming bilateral heart tubes, as well as in endoderm and ectoderm of the pharyngeal region. Sectioning revealed that, as the coelomic cavity was forming, *cNkx-2.5* expression was maintained in the thickened splanchnopleure (which at this axial level contains predominantly precardiac myocytes) but was absent from the somatopleure (Fig. 2J). At stage 10, strong *cNkx-2.5* was detected in the tubular heart (Fig. 2D). In sections of a whole-mount in situ from this stage (Fig. 2K), cardiac expression was confined to the myocardium (and not the endocardium), and *cNkx-2.5* was also expressed in the floor of the pharynx and pharyngeal ectoderm. By stage 14, *cNkx-2.5* expression was detected solely in the heart, as assayed by whole-mount in situ hybridization (Fig. 2E).

From stages 5 to 9, the mesodermal component of *cNkx-2.5* expression is remarkably coincident with fate maps of cardiac progenitor cells (Rosenquist and DeHaan, 1966), and thereafter it is found predominantly in the differentiated muscle of the heart. Because muscle-specific expression of *cNkx-2.5* is restricted to cardiac tissue (and absent from both smooth and skeletal muscle), expression of this gene in combination with other muscle differentiation markers is indicative of cardiac myogenesis.

A system for studying heart specification in the avian embryo

An experimental system was designed to study cardiac myocyte specification (schematized in Fig. 3A). The posterior-most section of the primitive streak (PPS) was dissected from a stage 4 chick embryo and divided longitudinally along the midline into two equivalent halves; this tissue is referred to as the responding tissue. Half of the stage 4 PPS was cultured alone as a control. The contralateral half was cultured in combination with potential inducing tissues taken from quail embryos. Cells within the PPS from stage 4 embryos have been fate mapped to give rise to extraembryonic tissues, including blood islands (Settle, 1954; Rosenquist, 1966; Schoenwolf et al., 1992), and are not destined to give rise to cardiac tissue. Indeed, prospective cardiac cells leave the primitive streak by stage 3c and are never found in the most posterior part of the streak (Garcia-Martinez and Schoenwolf, 1993).

After 48 hours in explant culture, the tissues were harvested for RNA, and gene expression was assayed by RT-PCR. By this time, control cultures taken from the prospective cardiac region in the AL mesendoderm of stage 4 embryos had been beating for approximately 24 hours. Primers were employed in the PCR analysis that amplified cDNAs encoding: (1) cNkx-2.5, whose expression pattern is described above; (2) cardiac troponin C (cTnC) which is expressed in cardiac and skeletal muscle (Maisonpierre et al., 1987; Toyota et al., 1989); (3) ventricular myosin heavy chain (vMHC) which is expressed primarily in cardiac ventricles, but also transiently in differentiating skeletal muscle (Bisaha and Bader, 1991); (4) ρ-globin which is an embryonic red blood cell marker (Dodgson et al., 1983); and (5) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which is expressed in a wide variety of cell types and used as a marker of tissue quantity (Panabieres et al., 1984; Weiskirchen et al., 1993). For each gene, primers were chosen that matched both the chick and quail genes. After PCR, each reaction product was incubated with a restriction enzyme which differentially cleaves the chick and quail amplification products. The digests were subsequently analyzed by polyacrylamide gel electrophoresis. This protocol allowed us to distinguish whether the above described genes were transcribed in either the chick or quail tissues (Fig. 3B). Importantly, the absolute amounts of chick and quail PCR products remained constant when amplified independently or in combination (Fig. 3B, compare lanes 2 and 3 with 4), indicating that the observed ratio of chick and quail PCR products reflects the actual ratio of cDNAs in the starting material.

Prior to performing induction experiments with PPS tissue, we evaluated cardiac gene expression in this tissue at the time of dissection. cNkx-2.5 and vMHC products were undetectable in non-cultured stage 4 PPS (Fig. 4A, lanes 7). However, trace levels of cTnC were present in stage 4 PPS (Fig. 4A, lanes 7). Because stage 4 PPS is outside of the fate map of the heart and explant cultures of this tissue failed to yield cardiac myocytes

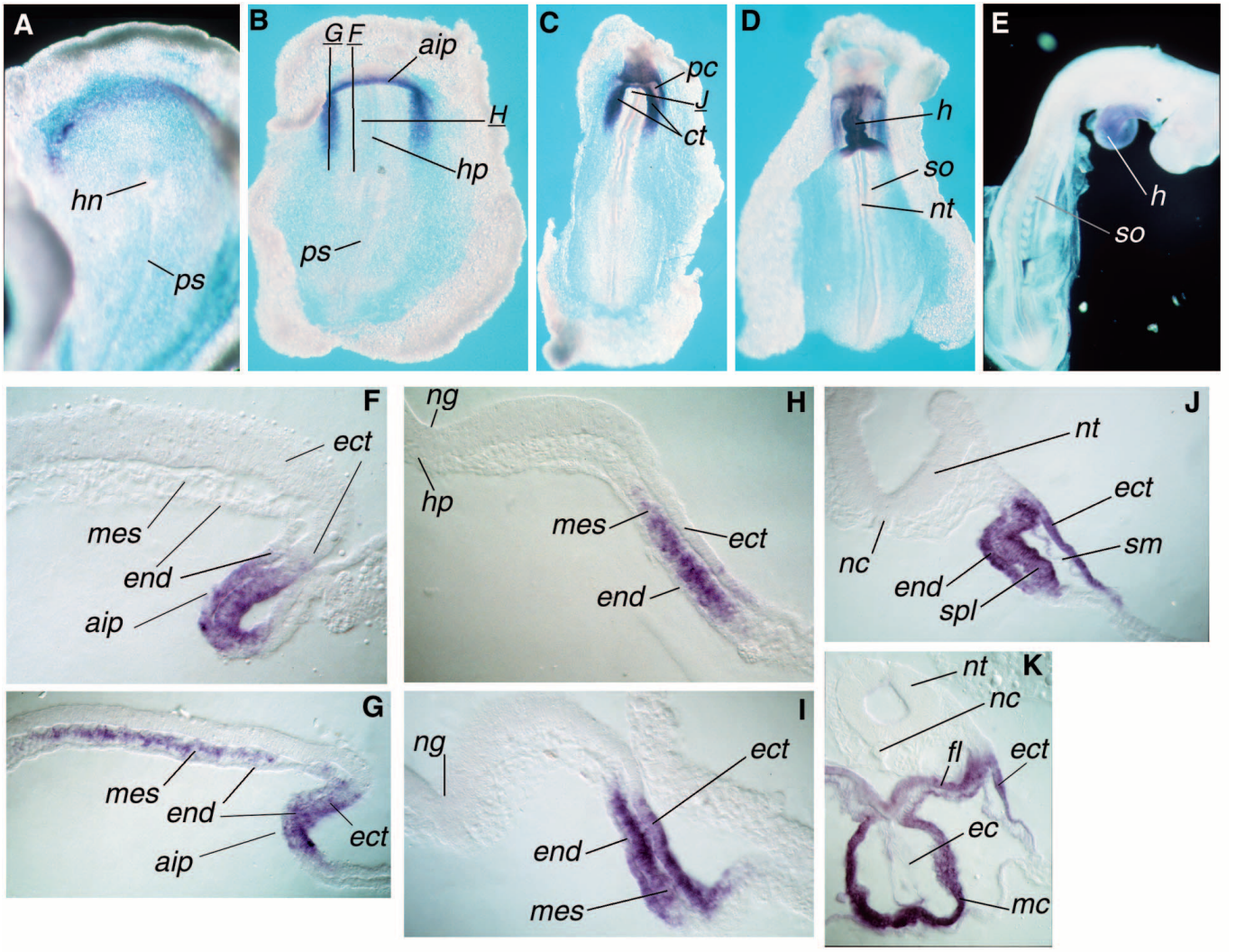

Fig. 2. Whole-mount in situ hybridization of chick embryos with antisense cNkx-2.5 digoxigenin-labelled probe. (A) Stage 5, dorsal view. Faint expression is found in a crescent in the anterior and lateral regions. (B) Stage 6, ventral view. Expression is seen lateral to the neural plate and in the region of the anterior intestinal portal. (C) Stage 8+, ventral view. cNkx-2.5 expression is detected in the forming cardiac tubes and in the pharyngeal region. (D) Stage 10, ventral view. The predominant signal is found in the tubular heart. (E) Stage 14, side view. cNkx-2.5 expression is detected only in the heart. (F) Longitudinal section of the anterior central region at stage 6 at the level indicated in B, anterior on the right. Expression is seen in the endoderm and ectoderm near the AIP, but not in the anterior central mesoderm. (G) Longitudinal section of the anterior lateral region at stage 6 at the level indicated in B, anterior on the right. Expression is seen in the anterior lateral mesoderm, as well as in the endoderm and ectoderm near the AIP. (H) Transverse section of stage 6, at the level indicated in B, lateral on the right. Expression is seen in the lateral plate mesoderm. (I) Transverse section of stage 7, at approximately the level of H, lateral on the right. Expression is seen in the lateral plate mesoderm as well as the adjacent ectoderm and endoderm. (J) Transverse section of stage 8+, at the level indicated in C. cNkx-2.5 expression is seen in the lateral splanchnic mesoderm, ectoderm and endoderm, but not in the somatic mesoderm. (K) Transverse section of stage 10, at the level of the heart. Expression is detected in the myocardium but not the endocardium and is also found in the floor of the pharynx and pharyngeal ectoderm. (aip, anterior intestinal portal; ct, cardiac tubes; ec, endocardium; ect, ectoderm; end, endoderm; fl, pharyngeal floor; h, heart; hn, Hensen's node; hp, head process; mc, myocardium; mes, mesoderm; nc, notochord; ng, neural groove; nt, neural tube; pc, pericardiac coelom; ps, primitive streak; sm, somatopleure; so, somite; spl, splanchnopleure.)

(see below), it seems that this early low-level *cTnC* expression takes place in the absence of cardiac myocyte specification.

Diffusible factors in the AL mesendoderm can induce cardiogenesis in posterior primitive streak explants

Stage 4 chick PPS was cultured with and without AL mesendoderm from stage 4-5 quail embryos. After 48 hours in culture, cNkx-2.5, cTnC and vMHC were strongly expressed in chick PPS explants in the presence, but not in the absence of quail AL mesendoderm (Fig. 4A, compare lanes 1, 3 and 5 with lanes 2, 4 and 6). Quail AL tissue also expressed all three cardiac muscle markers, which was expected since the AL mesoderm of stage 4-5 avian embryos is known to contain cardiac progenitor cells (Rosenquist and DeHaan, 1966). The amounts of chick GAPDH present in PPS cultured in the absence and presence of inducing tissue were similar, indicating that the inductive effect was not due to a general effect on

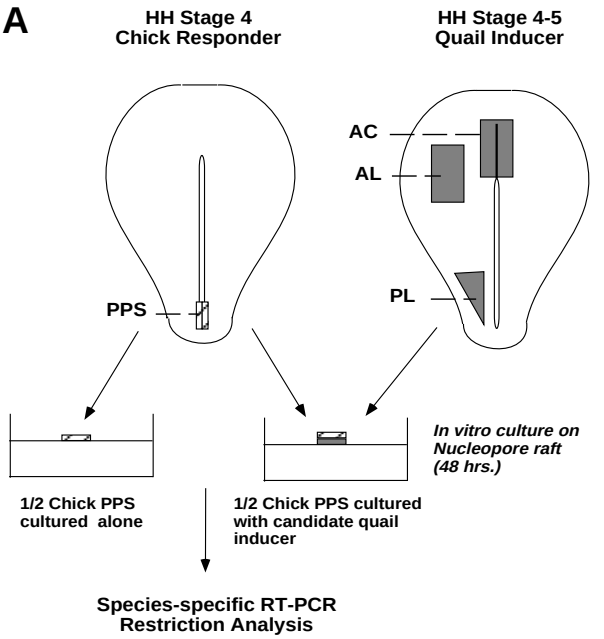

Fig. 3. Chick/quail co-culture system used for studying cardiac muscle specification. (A) Experimental design. Responder explants from the PPS of a stage 4 chick embryo were divided into two equal portions longitudinally. One half was cultured alone and the other half was cultured together with potential quail-inducing tissues. After 48 hours, tissues were harvested and species-specific RT-PCR and restriction analysis was performed as described in the text. (AC, anterior central; AL, anterior lateral; PL, posterior lateral; PPS, posterior primitive streak.) (B) Demonstration of species-specific RT-PCR and restriction analysis. Each set of PCR primers was used to amplify cDNA from embryonic day 3 chick heart (lanes 1 and 2), embryonic day 3 quail heart (lanes 3), or a mixture of the chick and quail heart cDNAs (lanes 4). In lanes 4, the starting concentration of chick and quail cDNA's was the same as when each of these cDNAs was amplified separately in lanes 1-3. PCR products were either analyzed directly (lanes 1) or first digested with restriction enzymes that differentially digest the chick and quail PCR products (lanes 2-4). For each gene there is at least one band diagnostic of the chick and quail gene products. Note that both chick and quail gene products can be recognized in a mixture of the two cDNAs (lanes 4). (C, chick; Q, quail; U, uncut.)

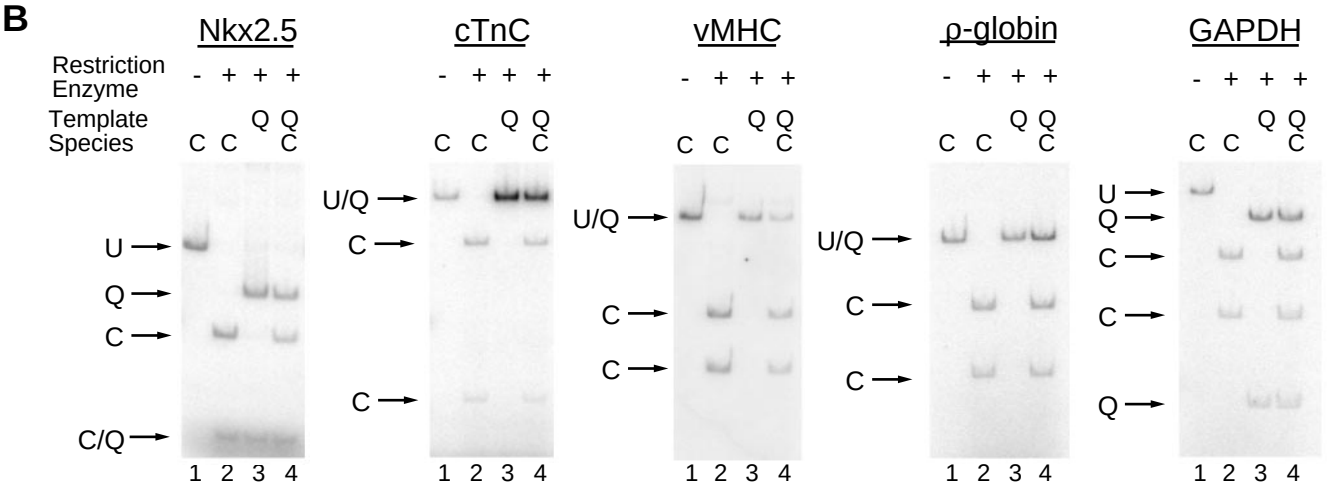

Table 1. Induction of vMHC and cTnC in coculture experiments

	vMHC			cTnC		
	Induction index			Induction index		
Inducer	> 10	2-10	< 2	> 10	2-10	< 2
AL ME	71% (15)	0% (0)	29% (6)	57% (21)	27% (10)	16% (6)
AL Meso	0% (0)	0% (0)	100% (11)	0% (0)	0% (0)	100% (11)
PL ME	0% (0)	0% (0)	100% (18)	0% (0)	56% (10)	44% (8)

The fold-induction of the cardiac muscle markers vMHC and cTnC in chick PPS co-cultured with the indicated quail inducing tissue is summarized. For vMHC, an 'induction index' was calculated which represents the ratio of chick vMHC levels, as determined by phosphorimager analysis, of individual PPS explants cultured with the indicated quail inducer tissue ('induced') versus the contralateral PPS explant cultured alone ('control'). Values were corrected for GAPDH levels (see formula). Table entries are given as a percentage of the number of explants examined (actual number in parentheses). In most instances, the signal in the control sample was zero, and hence any signal in the induced sample resulted in an induction index >10. If vMHC signals in both induced and control samples were zero, then the induction index was taken as zero. The cTnC induction index was determined in the same way, substituting 'cTnC' for 'vMHC'.

$$\text{vMHC induction index} = \frac{\text{(Chick vMHC-induced)/(Chick vMHC-control)}}{\text{(Chick GAPDH-induced)/(Chick GAPDH-control)}}$$

(AL ME = anterior lateral plate mesendoderm; AL Meso = anterior lateral plate mesoderm; PL ME = posterior lateral plate mesendoderm.)

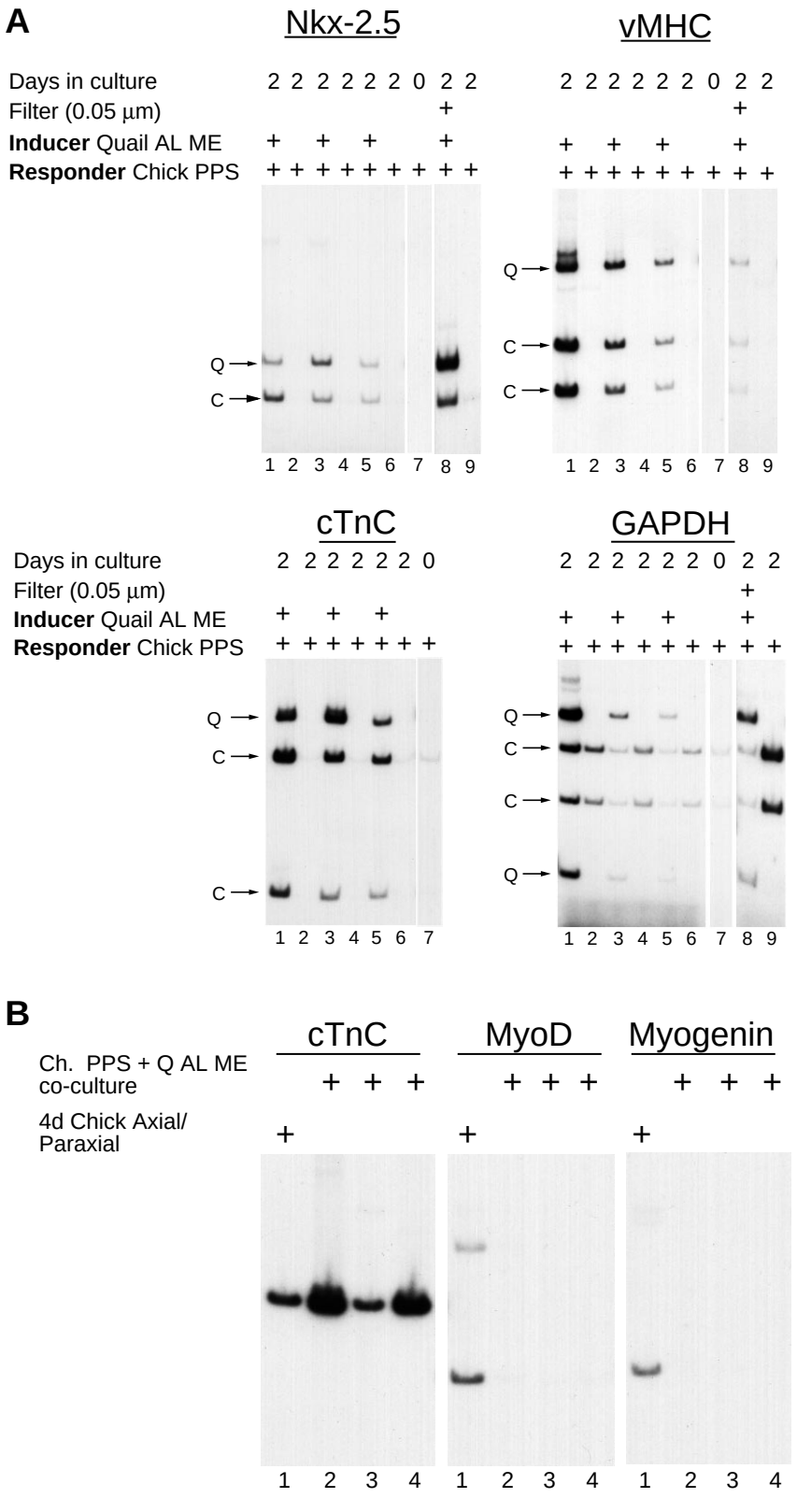

Fig. 4. (A) Anterior lateral mesendoderm induces expression of cardiac muscle markers in posterior primitive streak explants. Contralateral halves of individual stage 4 chick PPS explants were cultured either alone (lanes 2, 4, 6, 9), or together with stage 4-5 quail AL ME (lanes 1, 3, 5, 8). After 48 hours in culture, tissues were harvested and species-specific RT-PCR and restriction analysis was performed for cNkx-2.5, cTnC, vMHC and GAPDH. Expression of these genes was also evaluated in PPS tissue that was immediately harvested from stage 4 chick PPS without culturing (lanes 7). In lanes 8, a 0.05 μm Nucleopore filter was placed between inducer and responder tissues during the culture period. (B) Skeletal muscle is not present in co-cultures of PPS and AL ME. The expression of cTnC, MyoD, Myogenin and GAPDH was examined in three separate co-cultures of chick PPS and quail AL ME (lanes 2-4), and in immediately harvested trunk axial/paraxial tissues of an embryonic day 4 chick embryo, which contains differentiated skeletal muscle (lanes 1). The embryonic day 4 axial/paraxial tissue was diluted until its GAPDH signal was in the linear range and comparable to the GAPDH signals of the explant cultures. (C, chick; Q, quail; AL, anterior lateral; ME, mesendoderm; PPS, posterior primitive streak.)

PPS cell growth or survival. The results of a series of such cultures are summarized in Table 1. vMHC was induced greater than 10-fold in 71% of chick PPS explants when cultured with quail AL mesendoderm. Similar results were observed for cTnC: 57% of chick PPS explants showed a greater than 10-fold induction of cTnC when co-cultured with quail AL mesendoderm, and a further 27% showed a modest induction (2 to 10-fold). Similar results were seen when the age of the quail AL inducer varied from stages 3c to 6, or when the chick PPS responder varied from stages 2 to 5 (data not

shown). Placement of a 0.05 μm Nucleopore filter between the inducing and responding tissues did not block induction (Fig. 4A, lanes 8-9), indicating that the inductive interaction does not require cell-cell contact.

AL mesendoderm induces cardiac but not skeletal muscle

Because cTnC is also expressed in slow skeletal muscle (Maisonpierre et al., 1987; Toyota et al., 1989) and vMHC is transiently expressed in myotomal muscle (Bisaha and Bader, 1991), we investigated whether skeletal muscle was present in PPS tissue co-cultured with AL mesendoderm. The presence of skeletal muscle was assayed by monitoring the expression of the myogenic basic Helix-Loop-Helix (bHLH) genes MyoD and myogenin, which are expressed solely in skeletal muscle (Sassoon et al., 1989). MyoD is the first myogenic bHLH gene

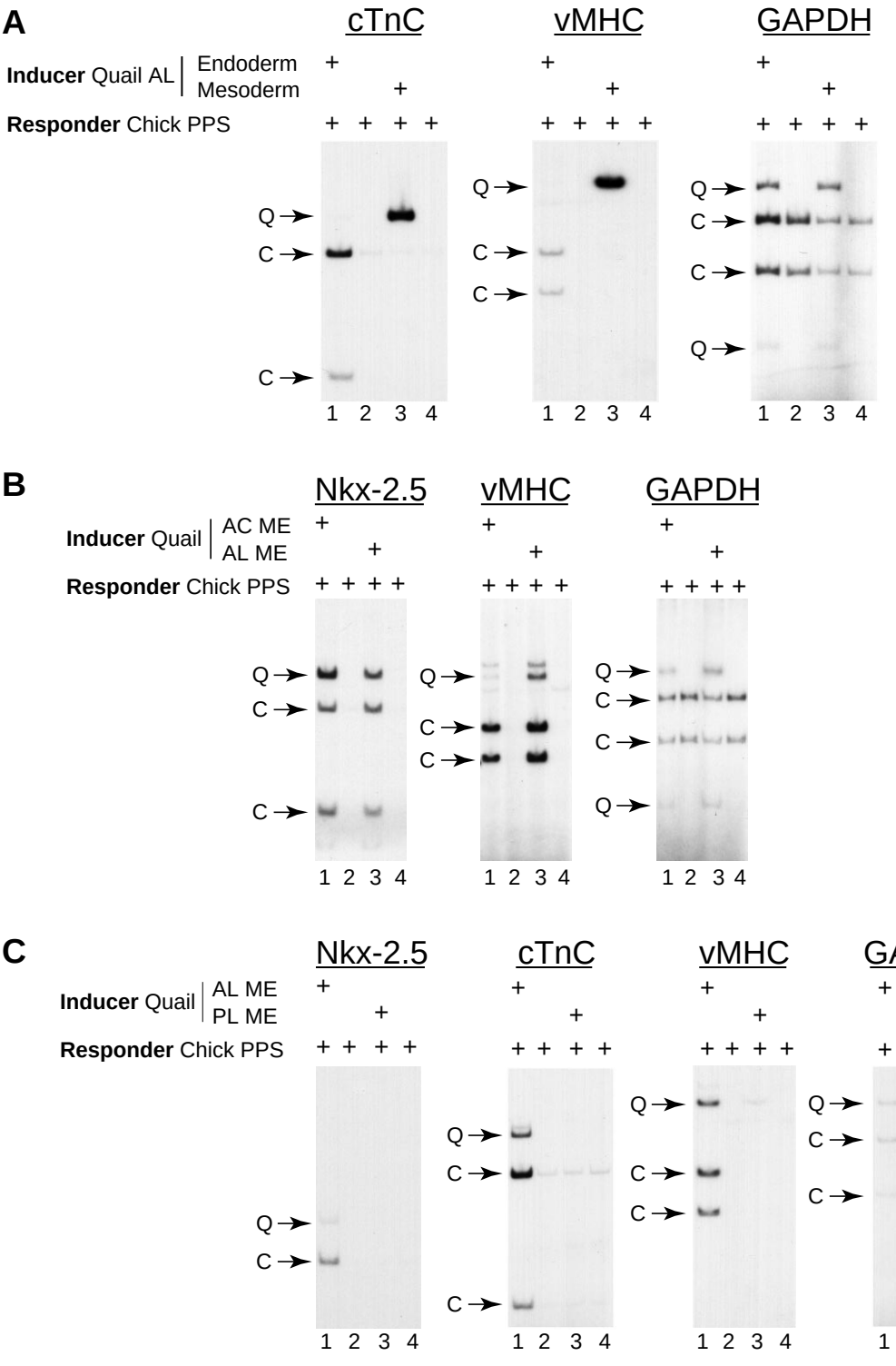

Fig. 5. (A) Cardiac-inducing activity is localized to the AL endoderm and is absent from AL mesoderm. Contralateral halves of individual stage 4 chick PPS explants were cultured either alone (lanes 2 and 4), or together with stage 5 quail AL endoderm (lanes 1) or mesoderm (lanes 3). After 48 hours in culture, tissues were harvested and species-specific RT-PCR and restriction analysis was performed for cTnC, vMHC and GAPDH. (B) Anterior central and anterior lateral mesendoderm possess equivalent cardiac-inducing properties. Contralateral halves of individual chick PPS were cultured either alone (lanes 2 and 4) or with stage 5 quail AC mesendoderm (lanes 1) or AL mesendoderm (lanes 3). After 48 hours in culture, tissues were harvested and species-specific RT-PCR and restriction analysis was performed for cNkx-2.5, vMHC and GAPDH. (C) AL ME is a much stronger cardiac inducer than PL ME. Contralateral halves of individual chick PPS were cultured either alone (lanes 2, 4) or with quail AL ME (lanes 1) or quail PL ME (lanes 3). After 48 hours in culture, tissues were harvested and species-specific RT-PCR and restriction analysis was performed for cNkx-2.5, cTnC, vMHC and GAPDH. The weak quail vMHC signal in lane 3 was not seen consistently. (C, chick; Q, quail; AC, anterior central; AL, anterior lateral; PL, posterior lateral; ME, mesendoderm; PPS, posterior primitive streak.)

expressed in avian embryos and myogenin is expressed in all myogenic cells at the onset of terminal differentiation (Pownall and Emerson, 1992). Neither MyoD nor myogenin was detectable in chick PPS/quail AL mesendoderm co-cultures (Fig. 4B, lanes 2-4), indicating that skeletal muscle was absent from these cultures and that all of the cTnC and vMHC gene expression was derived from induced cardiac myocytes.

The cardiac-inducing activity in the anterior lateral region is endoderm-specific

To further localize the cardiac-inducing activity in the AL mesendoderm, this tissue was separated into endodermal and mesodermal layers prior to culturing with chick PPS. Quail AL endoderm strongly induced chick cardiac gene expression in stage 4 chick PPS tissue (Fig. 5A, compare lanes 1 and 2); this endoderm was free from mesodermal contamination, as evidenced by the lack of quail cardiac gene expression. Conversely, quail AL mesoderm had no inductive effect, despite self-differentiating into quail cardiac muscle (Fig. 5A, lanes 3). Thus committed cardiac precursors are incapable of inducing PPS cells to become cardiac muscle. As summarized in Table 1, we have never observed induction of vMHC or cTnC in chick PPS co-cultured with quail AL mesoderm as the potential inducer. These results indicate that the active cardiac-inducing component in AL mesendoderm lies solely within the endoderm. Vigorous beating was observed in many cultures in which the inducing tissue contained no detectable quail cardiac muscle markers, indicating that AL endoderm can induce essentially complete cytological differentiation of cardiac myocytes from PPS explants.

Cardiac-inducing properties of the anterior central mesendoderm

Cardiac precursor cells are confined to the AL mesoderm of stage 5 and later embryos, and are not found in the anterior central (AC) region (Fig. 3A; see also Rosenquist and DeHaan, 1966). To determine whether this difference in the localization of precardiac myocytes is due to restriction of cardiac-inducing activity to the AL endoderm, stage 5 AL and AC mesendoderm were tested for their ability to induce cardiac muscle gene expression in PPS explants (mesendoderm was used as the inducing tissue because of the difficulty of isolating the fragile AC endoderm cleanly). AC and AL mesendoderm were equally effective inducers of cardiac gene expression in the chick PPS (Fig. 5B). Interestingly, the quail AL and AC mesendoderm differed with respect to expression of quail cardiac markers. While both AL and AC mesendoderm expressed high levels of cNkx-2.5, only AL mesendoderm expressed high levels of vMHC (Fig. 5B, compare lanes 1 and 3). Therefore, whereas both quail AC and AL mesendoderm can induce vMHC in chick PPS tissue, the AC mesoderm is not itself competent to express this gene (see Discussion).

Cardiac-inducing activity is biased towards anterior regions of the endoderm

To determine whether cardiac-inducing activity is a property of all mid-gastrula endoderm, or is instead localized within the embryo, cardiac-inducing activity was compared in anterior lateral (AL) and posterior lateral (PL) mesendoderm (see Fig. 3A). In contrast to the robust cardiac-inducing potential of AL mesendoderm, the PL mesendoderm showed either very weak or no induction of PPS cardiac gene expression (Fig. 5C, compare lanes 1 and 3). As summarized in Table 1, vMHC was not significantly induced in chick PPS co-cultured with quail PL mesendoderm, and beating tissue was never observed. Similarly, high level induction of cTnC (i.e. greater than 10-fold) was never detected when PL mesendoderm was employed as a potential inducer. However, in approximately half of the cases examined, a 2- to 10-fold induction of cTnC was observed in PPS cultured with PL mesendoderm, suggesting that this tissue may contain a low level of cardiac-promoting activity.

Cardiac myocyte induction of PPS cells correlates with inhibition of blood cell formation

Mesodermal cells from stage 4 PPS are fated to give rise to blood islands, blood vessels, mesenchyme and posterior extraembryonic tissues (Settle, 1954; Rosenquist and DeHaan, 1966; Schoenwolf et al., 1992). To determine whether the induction of cardiac myocytes in PPS is due to respecification of cells that would normally give rise to blood, we analyzed explant cultures for ρ-globin expression, a marker of primitive red blood cells (Dodgson et al., 1983). ρ-globin was not detectable in immediate harvests of stage 4 PPS (data not shown). PPS cultured alone contained high levels of ρ-globin RNA and no detectable vMHC, a cardiac muscle marker (Fig. 6, lanes 2,4,6 and 8). In contrast, chick PPS co-cultured with quail AL mesendoderm contained significantly reduced levels of ρ-globin and high levels of chick vMHC (Fig. 6, lanes 1,3 and 5). PL mesendoderm, which did not induce cardiac muscle, failed to decrease ρ-globin expression in chick PPS (Fig. 6, compare lanes 7 and 8).

As summarized in Table 2, quail AL mesendoderm repressed the expression of ρ-globin mRNA in 85% of chick PPS explants (in 38% of the cases to levels less than 10% of that found in PPS cultured alone). In contrast, chick PPS co-cultured with quail PL mesendoderm showed either constant or elevated levels of chick ρ-globin expression in all cases. Thus, signals from AL endoderm induce cardiac myogenesis in PPS cells with a concomitant decline in red blood cell formation.

Table 2. ρ-Globin expression in coculture experiments

	ρ-Globin induction index	
Inducer	< 1	> 1
AL ME	85% (11)	15% (2)
PL ME	0% (0)	100% (4)

A ρ-globin induction index was calculated, which represents the ratio of chick ρ-globin levels in PPS explants cultured with the indicated inducer tissue ('induced') versus PPS explants cultured alone ('control'). Values were corrected for levels of chick GAPDH. Table entries are given as a percentage of the number of explants examined (actual number in parenthesis). An induction index <1 indicates repression of ρ-globin, while an induction index >1 indicates induction of ρ-globin.

$$\text{ρ-Globin induction index} = \frac{\text{(Chick ρ-globin-induced)/(Chick ρ-globin-control)}}{\text{(Chick GAPDH-induced)/(Chick GAPDH-control)}}$$

(AL ME = anterior lateral plate mesoendoderm; PL ME = posterior lateral plate mesoendoderm.)

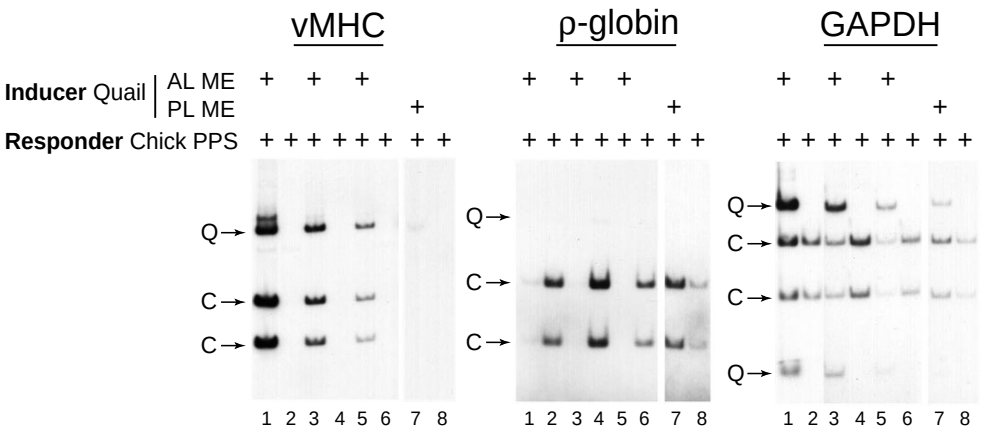

Fig. 6. Induction of cardiogenesis is accompanied by repression of a red blood cell marker. Contralateral halves of individual chick PPS were cultured either alone (lanes 2, 4, 6, 8), in the presence of quail AL ME (lanes 1, 3, 5), or in the presence of quail PL ME (lane 7). After 48 hours in culture, tissues were harvested and species-specific RT-PCR and restriction analysis was performed for vMHC, ρ-globin and GAPDH. (C, chick; Q, quail; AL, anterior lateral plate; PL, posterior lateral plate; ME, mesendoderm; PPS, posterior primitive streak.)

DISCUSSION

Signals from anterior endoderm induce cardiac myocyte specification in vitro

We have found that tissue from the avian mid-gastrula posterior primitive streak (PPS), which normally will give rise to blood islands, mesenchyme and extraembryonic tissues, will differentiate into cardiac muscle when co-cultured with anterior endoderm. This cardiac-promoting activity was specific to the anterior endoderm, being absent from AL mesoderm and greatly reduced in PL endoderm. When cultured alone, PPS explants differentiated into red blood cells (as indicated by high levels of ρ-globin expression) and contained comparable amounts of tissue in both the absence and presence of inducer. Thus, the cardiac-inducing effects of anterior endoderm cannot be attributed to a general effect on PPS cell viability and/or proliferation.

Several previous studies have suggested that endodermal tissues can promote heart differentiation in vertebrates (Jacobson, 1960, 1961; Jacobson and Duncan, 1968; Jacobson and Sater, 1988; Sugi and Lough, 1994; Nascone and Mercola, 1995). While these findings suggested that endodermal tissues could promote differentiation of the heart primordia, it was unclear whether signals from the endoderm were inducing myocardial specification or were instead promoting the differentiation of already committed cardiac myocytes. Our own findings indicate that, in avians, signals from the anterior endoderm can induce myocardial specification in cells normally fated to give rise to blood and extraembryonic tissue. These results suggest that anterior endoderm can influence cardiac myocyte lineage decisions and support previous results in amphibians suggesting that endoderm may play a role in this process (Bacon, 1945; Nascone and Mercola, 1995).

Our results indicate that anterior endoderm alone can induce cardiac myocyte differentiation in tissues that are not fated to give rise to heart. In contrast, Nascone and Mercola (1995) have recently reported that, in *Xenopus*, the deep endoderm underlying the heart rudiments is incapable of inducing heart tissue when combined with material from the ventral marginal zone (VMZ) of early gastrula embryos, but can induce heart formation in the VMZ when transplanted along with Spemann's organizer. These different tissue requirements for cardiac myocyte induction are consistent with several possible explanations: Firstly, there may be differences in the inducer tissues employed in the two sets of experiments. We used endoderm that was developmentally older than Nascone and Mercola (mid-late versus early gastrula). One possibility is that the organizer exerts its effect on cardiac myogenesis indirectly by patterning the endoderm, an event that may already have taken place in the avian AL endoderm by late gastrula stages. Secondly, there may be differences in the competence of the responding tissues employed in these two studies (i.e., *Xenopus* stage 10 VMZ versus avian stage 2-5 PPS). Although both tissues normally give rise to 'ventral' fates (i.e. red blood cells), the avian tissue is developmentally somewhat more advanced (mid versus early gastrula), and thus the avian stage 2-5 PPS may no longer require a second influence which, in the *Xenopus* VMZ experiments, is supplied by the organizer.

AC mesoderm has reduced competence to respond to cardiac inductive signals

Since heart muscle normally does not differentiate from AC mesoderm, it is of interest that the AC mesendoderm had strong cardiac-inducing properties in the co-culture assay. At least three explanations are consistent with these findings: AC mesendoderm may acquire cardiac-inducing activity in vitro (perhaps by being released from inhibitory influences of surrounding tissues) that it does not possess in vivo. Alternatively, cells in the AC mesoderm may be incapable of responding to cardiac-inducing signals, perhaps because of prior patterning by Hensen's node or axial structures. Finally, tissue in the AC mesendoderm (e.g. notochord) may produce signals that actively inhibit the response of the AC mesoderm to cardiac-inducing factors, although such inhibitory signals would have to be short range, since they do not inhibit the response of PPS to the AC mesendoderm. The observation that stage 5 quail AC mesendoderm did not express high levels of vMHC, despite inducing this and other cardiac muscle markers in co-cultured chick PPS, supports the second and/or third of these possibilities. Jacobson and colleagues have reported an inhibitory influence of neural plate on cardiac differentiation in amphibians (Jacobson, 1961; Jacobson and Duncan, 1968). However, there is no neural plate present in our experiments. If prospective neural tissues are involved in restricting the ability of AC mesoderm to respond to cardiac-inducing signals, then such influences would have to operate prior to stage 5.

Cardiac myocyte specification is accompanied by repression of erythrocyte differentiation

Several possible mechanisms could explain why production of cardiac muscle in stage 4 PPS is accompanied by a marked repression of the erythrocyte differentiation program. The PPS may contain multipotent cells capable of giving rise to both cardiac myocytes and erythrocytes. In this case, anterior endoderm would be effecting a lineage decision by the multipotential PPS cells. Alternatively, the PPS may contain at least two progenitor populations, capable of giving rise to myocardial and erythrocyte lineages, respectively. In that case, the anterior endoderm would be promoting the replication and survival of one class of progenitors (precardiac) and simultaneously inhibiting the replication and survival of the other class (preerythrocyte); see Holtzer et al. (1983) and references therein for additional discussion of this issue. Distinguishing between the possible mechanisms will require identifying markers for precursors to these lineages while they are still in the primitive streak.

Does anterior endoderm play a role in specification of cardiac myocytes in vivo?

At stage 3a-b, prospective cardiac tissue is located within and near the anterior primitive streak (Rosenquist and DeHaan, 1966; Garcia-Martinez and Schoenwolf, 1993). It appears that prospective myocardial cells from a stage 3 embryo are not completely specified, as they will not differentiate into cardiac myocytes when cultured as individual cells (Montgomery et al., 1994; T. S., unpublished data). By stage 4 to 4+, when the prospective cardiac myocytes have left the primitive streak and are located in the AL mesoderm (Rosenquist and DeHaan, 1966), individual cells or groups of AL mesodermal cells have become specified and will express cardiac myocyte markers in tissue culture in the absence of any apparent endoderm (Biehl et al., 1985; Antin et al., 1994; Montgomery et al., 1994; Gannon and Bader, 1995). Thus, an important stage in avian cardiac myocyte specification appears to take place between stages 3 and 4+. During this approximately 6 hour period, presumptive myocardial cells leave the primitive streak and come into contact with the AL endoderm. Because anterior endoderm from these stages possesses cardiac-inducing properties (this study), it seems plausible that AL endoderm may play a role in myocardial cell specification in vivo.

Our in vitro co-culture experiments, combined with the results of previous fate-mapping and culture experiments (Rosenquist and DeHaan, 1966; Garcia-Martinez and Schoenwolf, 1993; Montgomery et al., 1994), indicate that from early to mid gastrula phases (stages 3-5), both the anterior and posterior primitive streak contain cells that possess the potential of giving rise to cardiac muscle. Whether or not they do so may depend on which tissues they contact as they leave the primitive streak and migrate laterally. We propose (Fig. 7) that ingressing mesodermal cells from the early anterior primitive streak are induced to become cardiac tissue when they encounter AL endoderm (Pathway 2); more posterior cells in the primitive streak do not encounter endoderm with cardiac-inducing capacity and therefore give rise to tissues other than heart (Pathway 1). Mesodermal cells that reach the AC region (Pathway 3) are subject to competing patterning influences, most likely from midline structures, which inhibit the ability of these cells to respond to the cardiac-inducing signals of the AC endoderm.

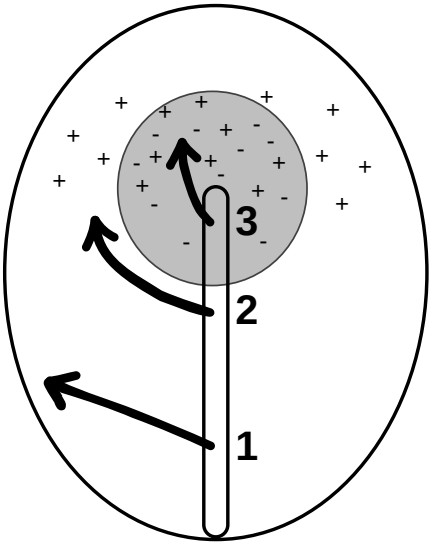

Fig. 7. A model for cardiac myocyte induction. Migration paths of mesoderm at various levels of the primitive streak are schematically indicated by arrows 1, 2 and 3. Invaginating mesodermal cells encounter signals that induce cardiac myogenesis in the anterior endoderm (indicated by '+') and signals which inhibit cardiac myogenesis in the anterior central mesendoderm (shaded and indicated by '–'). Invaginating cells that encounter neither signal (pathway 1) differentiate into non-cardiac fates, including blood. Cells that migrate into the anterior lateral plate (pathway 2) encounter only positive signals for cardiac myogenesis and differentiate into heart. Cells that migrate into the anterior central region (pathway 3) encounter inhibitory signals that prevent cardiac myocyte development.

Mesodermal expression of *cNkx-2.5* overlaps the cardiac fate map

Both the sequence and the expression pattern of the chick homeobox gene *cNkx-2.5* are highly related to that of the *Drosophila* gene *tinman*, mutations in which cause a failure in the formation of the dorsal vessel (the *Drosophila* heart) and visceral mesoderm (Bodmer, 1993). Previous studies have demonstrated that the Nkx-2.5 genes are expressed in cardiac myocytes and their precursors in mouse and *Xenopus* (Komuro and Izumo, 1993; Lints et al., 1993; Tonissen et al., 1994). In chick, the mesodermal expression pattern of *cNkx-2.5* RNA from stage 5 onward agrees to a remarkable extent with the fate map of the heart produced by Rosenquist using thymidine labelled grafts (Rosenquist and DeHaan, 1966). *cNkx*-2.5 appears to be the earliest reported marker of avian cells that are fated to become cardiac myocytes, appearing at least six hours prior to the earliest described myocardial structural protein (Han et al., 1992). At stage 5, when *cNkx-2.5* is first expressed, some myocardial cells have already become specified (Antin et al., 1994; Montgomery et al., 1994), suggesting that *cNkx-2.5* expression may initially arise in specified and/or committed but not yet differentiated cardiac myocytes. It should be noted however, that not all cells that express *cNkx-2.5* are fated to become cardiac myocytes as this gene, like the mouse gene (Lints et al., 1993), is also expressed in a limited

region of the ectoderm and endoderm. Therefore, transient expression of *cNkx-2.5* is apparently not sufficient to induce cardiac myogenesis.

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Note added in proof

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X91838.