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DIFFERENTIAL DISPLAY OF mRNAs FROM THE ATRIOVENTRICULAR REGION OF DEVELOPING CHICKEN HEARTS AT STAGES 15 AND 21

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ABSTRACT

In an effort to isolate novel genes that may be involved in the development of the cardiac cushions and then the formation of cardiac valves and septa, we utilized the differential mRNA display method in conjunction with the whole-mount *in situ* hybridization. The total RNA used for differential display was prepared from atrioventricular (AV) canal regions of stage 15 and stage 21 chicken hearts because critical events known to be important for the AV valve and septum formation occur during this period of the development. We have successfully obtained 14 potential candidate genes. Three examples, 15H16 (phospholamban), E13 (skeletal α-tropomyosin) and 21C (a novel gene), are discussed here. Levels of mRNA expression in developing hearts were determined by Northern blot analysis and their expression patterns were revealed and compared using whole-mount *in situ* hybridization. Both phospholamban and skeletal α-tropomyosin messages in the myocardium of the AV canal region showed significant decrease during this period of the development. The 21C differential display product detects a novel 9.5 Kb message whose expression is cardiac-specific at early stages of development. The expression level of the 21C gene appeared to be increased from stage 15 to stages 21 and 25 as determined by both Northern blot analysis and *in situ* hybridization. From these data, we demonstrate that the differential display method together with the whole-mount *in situ* hybridization could be an effective means for the isolation of novel and differentially expressed genes.

INTRODUCTION

The molecular mechanisms for cardiac morphogenesis are not completely understood. In chicken development, it has been shown that presumptive cardiac forming cells migrate anteriorly and medially, and then the right and left regions of these cells (lateral plate mesoderm) fuse at the midline to generate the primitive heart tube at stage 10 which starts to beat almost immediately (1).

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The cell lineage studies of the development of zebrafish embryos have led to the identification of cardiac forming regions in the early blastula (2). However, little is known about specific genes involved in the early cardiac morphogenesis except for several structural genes, such as ventricular myosin heavy chain (3), atrial myosin heavy chain (4), cardiac troponin I (5) and vascular smooth muscle α-actin (6), as well as few regulatory genes, such as MEF2 (7) and Drosophila homeobox gene tinman (8) and its mouse homologue Csx/Nkx-2.5 (9,10). In an effort to identify genes that are differentially expressed in embryonic hearts at the time of the formation of endocardial cushions, the progenitors of the valves and membranous septa, we have employed the differential mRNA display method (11) in this study. Since the differential display is highly sensitive, and likely to detect a large number differentially expressed genes, we have focused on comparing differences of mRNA expression between stage 15 and stage 21 of the atrioventricular (AV) canal regions of developing chicken hearts. It has been demonstrated that the epithelial-mesenchymal cell transformation occurring between stage 15 and 21 may be a key process during the AV cushion morphogenesis (12). Furthermore, this cell transformation appears to be induced by local stimuli derived from the adjacent myocardium (13-15). Thus, genes which are differentially expressed in the AV canal regions of stages 15 and 21 embryonic hearts may be important for the formation of the cardiac valves and septum. The cloned gene fragments we isolated using differential display were sequenced and compared against sequences in the GenBank database. In addition, cloned cDNA fragments were used as probes in whole-mount *in situ* hybridization. In total 14 differentially expressed fragments were cloned. Here, we report three examples of such clones: 15H16 for cardiac phospholamban, E13 for skeleton α-tropomyosin and 21C for a novel gene with a message size of 9.5 Kb.

MATERIAL AND METHODS

Tissue Collection and Total RNA Isolation

Fertilized White Leghorn chicken eggs (Hy-Vac, Adel, Iowa) were incubated at 38^{o}C in a humidified incubator with automatic rotation. All embryos were staged according to Hamburger and Hamilton (16). Hearts of stage 15 and stage 21 embryos were quickly dissected and tissues from atrioventricular (AV) canal region were collected and

frozen in liquid nitrogen, then stored in -70^{o}C freezer, or immediately used for isolating total RNA. Total cellular RNA was isolated either by using Tri Reagent RNA isolation kit (Molecular Research Center, Inc., Cincinnati, OH) according to manufacturer's manual, or by using guanidinium thiocyanate method (17,18). Total RNA was also prepared from both stage embryos without hearts and used for the comparison of differentially expressed genes.

Differential mRNA Display

Before use, total RNA samples were treated with RNase-free DNase I (Promega, Madison, WI) to remove potential chromosomal DNA contamination. About 2μg each of total RNA from stage 15 and stage 21 AV canal tissues or embryos without hearts were used for reverse transcription. Reverse transcription and subsequent PCRs were performed as previously described (11,19) with some modifications. The PCR cycling parameters were as follows: 94^{o}C for 45 sec, 42^{o}C for 1 min and 30 sec, 72^{o}C for 45 sec for 30 cycles followed by 72^{o}C for 5 min The PCR products together with size markers of known sequence were then separated on a 6% denaturing polyacrylamide gel. Differentially expressed gene fragments detected in either stage 15 or stage 21 AV canal tissues were identified and further compared to the displayed patterns obtained from stage 15 and stage 21 embryo tissues without hearts. Only those fragments with clear differential expression and not detected in embryos lacking hearts were selected for further cloning and characterization. The sequencing gel was run to resolve cDNA fragments sized from 200bp to 500bp. Bands of interest were cut out from the gel and cDNA fragments eluted by boiling the gel pieces in 100μl H_2O for 15 min were used as templates for PCR re-amplification using the same condition as described above. A portion of the re-amplified PCR product was analyzed on a 2% agarose gel to check the efficiency of re-amplification and to confirm the size of cDNA fragments. All primers used for reverse transcription and PCR were synthesized by Oligo Etc. Inc. (Guilford, CT). Primers used included: AP1 (T_{11}CA), AP2 (T_{11}GC), AP3 (T_{11}AG), AP6 (T_{11}AA), AP8 (T_{11}AT), AR1 (CTGATCCATG), AR2 (CTTGATTGCC), AR3 (CTGCTCTCAA). AmpliTaq DNA polymerase was purchased from Perkin-Elmer Corp. (Norwalk, CT.). α-[^{35}S]dATP (1200Ci/mmole) was obtained from New England Nuclear (Boston, MA.).

cDNA Cloning and Sequencing

Re-amplified cDNA fragments were cloned into either pBluescript II SK (pBK) vector (Stratagene, San Diego, CA) or the pCRII vector using the TA cloning system from Invitrogen (San Diego, CA). For cloning into pBluescript II SK vector, the vector DNA was linearized at the EcoRV site and a T residue was tailed to the blunt ends according to Marchuk *et al*.(20) to facilitate the direct cloning of PCR fragments. DNA sequencing was performed using Sequenase Kit Version 2.0 (United States Biochemical, Cleveland, OH). The nucleotide sequences obtained were compared with GenBank databases using the GCG (Genetics Computer Group) FASTA program software (Madison, WI).

Northern Blot Analysis

For Northern blot analysis, total RNA was isolated from stage 15 and stage 21 chicken hearts. 10 μg of total RNA was loaded on a 0.8% formaldehyde/agarose gel. After electrophoresis, the RNA was eletrophoretically transferred to Zetaprobe nylon membrane (Bio-Rad, Richmond, CA). DNA probes were labeled either by nick translation (Promega, Madison, WI) for longer cDNA fragments or by radioactive PCR-labeling protocol (21) for shorter fragments. Hybridization and washes were performed as described previously (21).

PhosphorImager Analysis

After hybridization and washing, the Northern blot membranes were exposed to a PhosphorImager plate (Molecular Dynamics, Sunnyvale, CA.). The radioactivity on the plate was scanned and analyzed by a PhosphorImager 450 (Molecular Dynamics, Sunnyvale, CA.).

Whole-Mount *In Situ* Hybridization

For the *in situ* hybridization, plasmids containing cDNA fragments in either pBK or pCRII vector were used as templates for the preparation of digoxigenin(DIG)-labeled riboprobes. DIG-labeling kit was purchased from Boehringer Mannheim (Indianapolis, IN) and riboprobes were synthesized according to the manufacturer's protocols. Both sense and antisense DIG-labeled RNA probes were synthesized by the *in vitro* transcription of linearized DNA templates, in the presence of either SP6, T3, or T7 RNA polymerase using DIG-labeled uridine-triphosphate (DIG-UTP) as one of nucleotide substrates.

Whole-mount *in situ* hybridization was performed generally according to the methods of Hemmati-Brivanlou *et al*.(22) and Riddle *et al*. (23) with some modifications. Briefly, staged chicken embryos were dissected in PBS buffer and fixed in M buffer (100mM MOPS pH 7.4, 2mM EGTA, 1mM $MgSO_4$, and 3.7% formaldehyde) at 4^{o}C overnight. After bleaching in M buffer with 6% hydrogen peroxide for 48 hr at room temperature, embryos were dehydrated through an ascending methanol series in PBS (25, 50, 75 and 100% methanol) and stored in 100% methanol at -20^{o}C until needed.

Embryos were rehydrated through a descending methanol series in PBT (PBS plus 1% Tween 20) and PBT, permeabilized in PBT containing proteinase K (μg/ml in PBT) for 30 minutes at room temperature. After post-fixing in 4% paraformaldehyde in PBT, embryos were washed three times in PBT, rinsed once each in 0.1M triethanolamine (pH 8) and in 0.1M triethanolamine plus acetic anhydride (2.5l/ml). After incubation with prehybridization solution (0.3M NaCl, 20mM Tris pH 8.0, 1mM EDTA, 0.1M DTT, 1X Denhart's, 10% dextran sulfate, 50% formamide, 250g/ml yeast tRNA, and 100g/ml salmon sperm DNA) for 2 hr at 50°C or 60°C, embryos were hybridized with DIG-labeled riboprobes in hybridization solution (prehybridization solution plus 0.5-1μg/ml riboprobe) at 50°C or 60°C overnight. After hybridization, embryos were washed two times (30 min each) with prehybridization solution at the hybridization temperature, followed by washing two times (30 min each) with 50% formamide, 2X SSC at the same temperature. Embryos were then treated with RNase A (1μg/ml) and RNase T1(1 unit/ml) for 30 min at 37°C. After a final wash with 0.2X SSC, 1% Tween 20 at 50°C or 60°C, embryos were incubated with PBT plus 10% heat inactivated sheep serum (Sigma, St.Louis, MO) for 2 hr at room temperature and then with alkaline phosphatase conjugated Fab anti-DIG solution (1 to 2000 dilution in PBT plus 10% sheep serum) overnight at 4°C. Embryos were washed at least 8 times with PBT (1 hour each) and left in PBT overnight at 4°C to completely wash out excess amounts of alkaline phosphatase conjugated Fab. Color reaction was performed by washing embryos three times for 10 min each with buffer A containing 100mM Tris pH 9.5, 50mM $MgCl_2$, 100mM NaCl, 1% Tween 20, and 2mM levamisol, followed by incubating with detection solution (buffer A plus 4.5μl/ml nitroblue tetrazolium and 3.5μl/ml 5-bromo-4-chloro-3-indolyl-phosphate toluidinium) for various times at room temperature. When the color reaction was complete, embryos were washed twice with buffer A, once with PBT, and then post-fixed in 4% paraformaldehyde in PBT. After several washes with PBT, embryos were observed and photographed with a Wild M420(Swiss) stereo microscope using either bright-field or dark-field optics with either Kodak Ektar 64 or Royal Golden 100 film. Selected embryos were embedded in Paraffin Plus (Oxford, St. Louis, MO) and sectioned at 10-15μm. After deparaffinizing, rehydrating and mounting on Super frost/Plus slides (Fisher), sections were photographed with bright-field or phase-contrast optics using a Leitz Laborlux 12 microscope (Leitz Wetzlar, Germany) and Kodak Royal Golden 100 or 25 films.

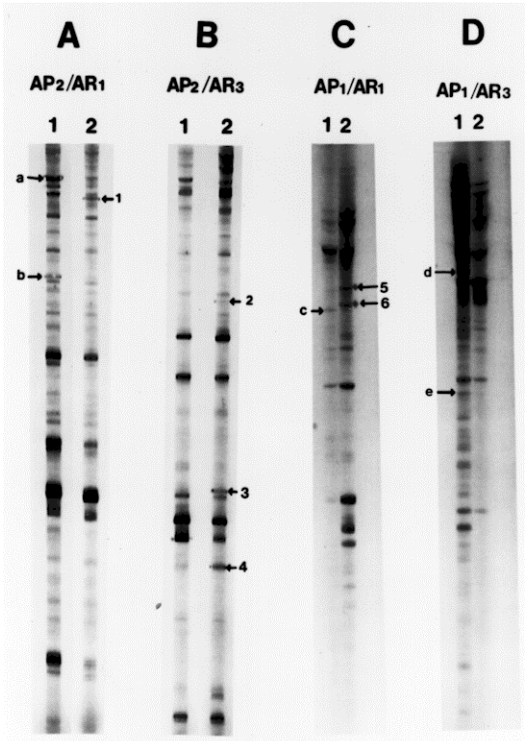


Figure 1 Differential display of mRNAs from AV canal regions of stage 15 versus stage 21 embryonic hearts. Total RNA isolated from AV canal regions of stage 15 (lane1) and stage 21 (lane2) chicken embryonic hearts were reverse-transcribed using either AP1 or AP2 oligo-dT primer. Samples were further PCR amplified by the addition of either arbitrary AR1 or AR3 primer as described under the materials and methods. The amplified products were displayed side-by-side on a 6% sequencing gel. Several candidate cDNA fragments were differentially expressed in stage 15 (marked by a-e) or stage 21 (marked by 1-6). Note that band 4 was one of the cDNA fragments (described below), which represented a novel gene, 21C, identified in this study.

RESULTS

Differential display of mRNAs from AV canal regions of stage 15 and stage 21 hearts

Different combinations of five (T_{11})MN anchoring primers and three arbitrary 10-mers were used in the differential display. Of all 15 possible primer combinations, four (AP1/AR1, AP1/AR3, AP2/AR1, AP2/AR3) displayed reproducible and differential patterns of expression (Fig 1). The differentially expressed cDNA fragments were readily identified from either stage 15 sample (bands a-e of Fig 1) or stage 21 sample (bands 1-6 of Fig 1). In

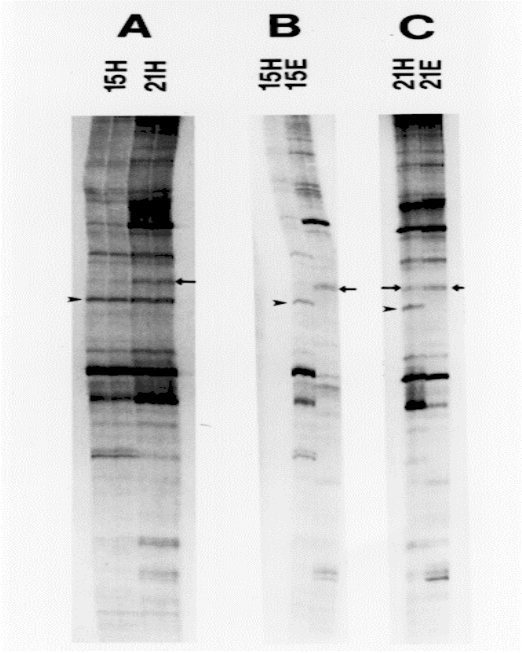


Figure 2 Differential display of mRNAs from stage 15 and stage 21 hearts versus stage 15 and stage 21 whole embryos without hearts. Total RNA isolated from stage 15 chicken embryonic hearts (15H) and embryos without heart tissues (15E), as well as stage 21 chicken embryonic hearts (21H) and embryos without heart tissues (21E) were used for reverse-transcription and PCR amplifications. Primer sets used were AP2 and AR3. Many differentially expressed cDNA fragments detected in RNA from heart tissue (A) were not significantly different in RNA from whole embryos without hearts between stage 15 and stage 21 (B and C, indicated by an arrow). On the other hand, some fragments appeared to be cardiac-specific but were not differentially expressed (indicated by arrowhead).

addition, there existed many cDNA fragments showing slightly differential expression.

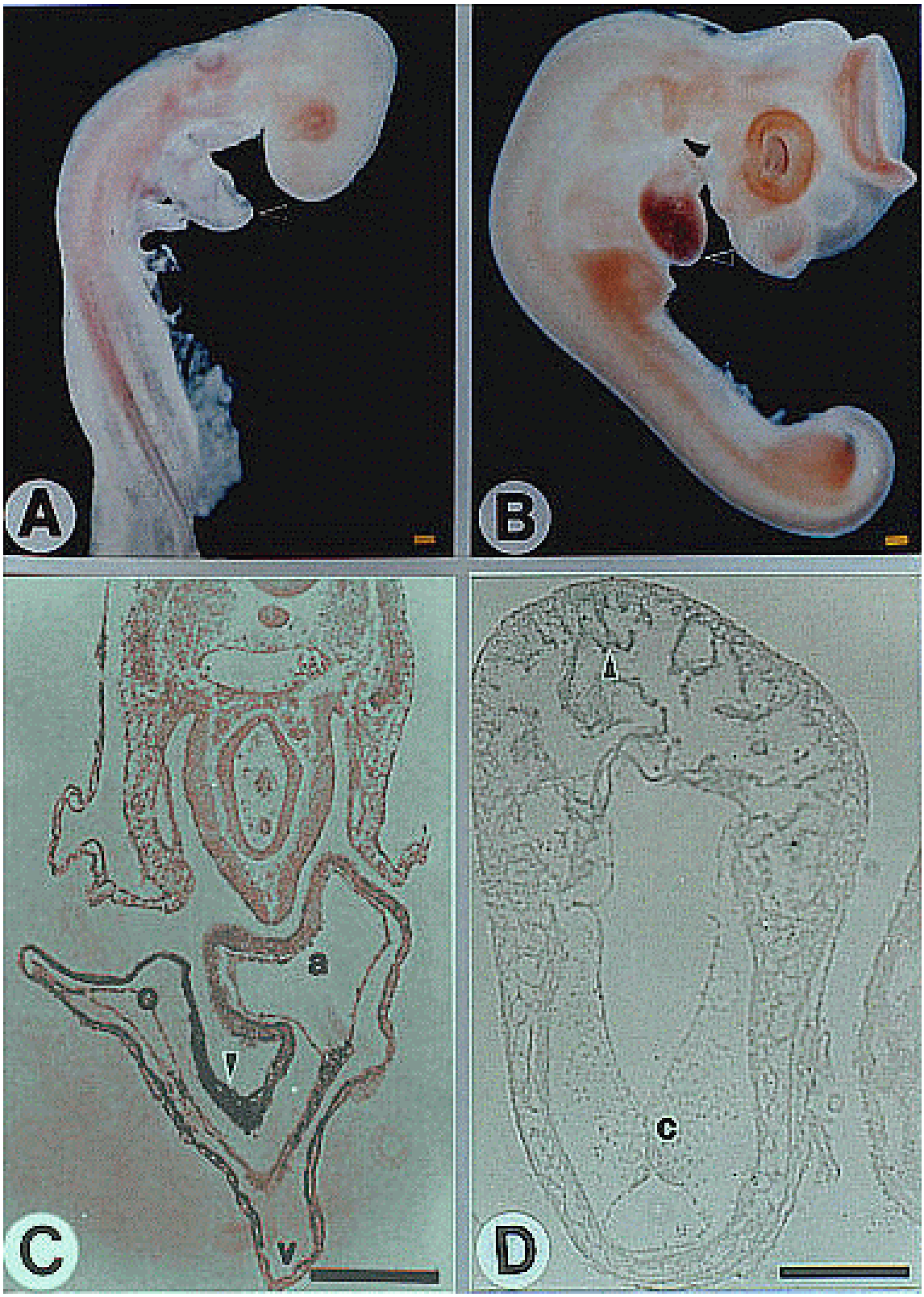
To identity cardiac-specific differential expression between stage 15 and stage 21, we also displayed cDNAs prepared from RNA samples isolated from the same staged embryo tissues without hearts. Examples of such display and comparison are shown in Fig 2. One of cDNA fragments indicated by arrow in Fig 2A could be thought as a cardiac-specific and differentially expressed gene if just compared to the differential expression patterns from heart tissue. By the addition of differential expression patterns generated from the same stage embryos without hearts (Fig 2B and 2C), such false positive clone could then be ruled out, because of the presence of an equally expressed band (indicated by arrows in 15E and 21E of Fig 2B and 2C). Conversely, cardiac-specific but not differentially expressed genes could be readily identified by comparisons of displaying patterns from heart and embryo without heart (indicated by arrowheads in Fig 2).

From the displaying sequencing gels, we cut out over 20 different bands, ranging in size from 200bp to 500bp. These bands were subcloned and their sequences were determined. Table 1 summarizes 14 cDNA clones. From the sequence comparison to the GenBank database, 4 clones represent known genes, including protein synthesis initiation factor eIF-4AII (clone 15A), phospholamban (clone 15H16), skeletal α-tropomyosin (E13) and α-actin (clone H2). These cloned cDNA fragments were further used as probes in the whole-mount *in situ* hybridization screening. Of 12 clones tested, 10 probes show specific hybridization to the heart and only 2 probes did not detect significant signal. This is in contrast to the evaluation of cloned cDNA fragments by Northern blot analysis. The rate of success in identifying message sizes by Northern blot analysis with these cDNA probes was very low (29%). We chose 3 clones for further evaluation of their expression patterns in the AV canal regions of stage 15 and stage 21 hearts.

Phospholamban is differentially expressed in AV canal regions of developing hearts between stage 15 and stage 21

From the DNA sequence, we found that the clone 15H16 contained 320bp upstream from the poly(A) tail of the chicken phospholamban transcript (24). Phospholamban is a protein regulating the

Figure 3 Expression patterns of 15H16 clone (chicken cardiac phospholamban) in stage 15 and stage 21 embryos by *in situ* hybridization. Whole-mount *in situ* hybridization with stage 15 (A) and stage 21 (B) embryos were performed using DIG-UTP labeled 15H16 antisense riboprobe. After whole-mount analysis, cross sections through heart regions of the same embryos were also examined (C and D). Note that the 15H16 gene was exclusively expressed in developing hearts (arrowheads in A and B) of stage 15 and stage 21 embryos. Cross-section through the stage 15 heart (C) revealed that the 15H16 message was restricted to myocardium with a high level of expression in the myocardia of outflow tract and AV canal region. On the other hand, cross-section through the stage 21 heart (D) showed that the 15H16 message was confined to the trabecular myocardium but not to the AV or outflow tract myocardium. (A and B) dark-field micrographs, (C and D) bright-field micrographs. a, atrium; c, cardiac cushion; o, outflow tract; v, ventricle. Scale bar = 200μm.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sarcoplasmic reticulum Ca^{++} pump activity and thus, plays an important role in the contraction and relaxation of cardiac and slow skeletal muscles (24-27). Although the expression pattern of phospholamban in developing recently reported in paraffin-sections by *in situ* hybridization (26,27), its expression in developing AV canal region remained unclear. The cloned cDNA fragment 15H16 was obtained from RNAs isolated from stage 15 AV canal region by differential display method, suggesting a decreasing expression of phospholamban in the AV region from the stag 15 heart to the stage 21 heart. However, the whole-mount *in situ* hybridization showed that phospholamban mRNA signals were stronger in the stage 21 heart (Fig 3B) than in the stage 15 heart (Fig 3A). The overall increase in phospholamban messages between these two hearts has been developing stage hearts was consistent with the previous reports by Toyofuku *et al*. (26) and Toyofuku and Zak (24). When spatial expression patterns of phospholamban were examined in cross-sections from the same embryos after whole-mount *in situ* hybridization, we detected a drastic decrease in the phospholamban expression at the AV canal myocardium and the outflow tract myocardium from stage 15 heart (Fig 3C) to stage 21 heart (Fig 3D). Northern blot analysis of total RNAs isolated from stage 15 and stage 21 hearts revealed that two bands with sizes of 1.1Kb and 0.6Kb hybridized to the 15H16 probe (Fig 4A). The steady-state levels of these two messages increased from stage 15 to stage 21. Therefore, the overall expression of phospholamban in the heart increased from stage 15 to stage 21, although its expression at the AV canal region appeared to be decreased.

Skeletal α-tropomyosin expression is temporally and spatially decreased in the developing hearts

The clone E13 was obtained from stage 15 RNA using the differential display method. E13 contained 268bp upstream from the poly(A) tail of chicken skeletal α-tropomyosin transcript (32). In avians, cardiac α-tropomyosin has been shown to differ from the skeletal α-tropomyosin in respect to its mobility in 2D gels (28,29) and at the nucleotide sequence (30-32). As can be seen in Fig 5, skeletal α-tropomyosin messages were detected in developing somites (open arrows) and hearts (arrowheads) at both stage 15 and stage 21 embryos. The hybridization signal was strong in the stage 15 heart and became weaker in the stage 21 heart. On the other hand, the signals became much stronger in the stage 21 somites (Fig 5A and 5B). Examination of cross sections of the heart of the same embryos revealed that E13 was evenly expressed in the myocardium of stage 15 heart (Fig 5C). As the development proceeded, the expression of E13 in the

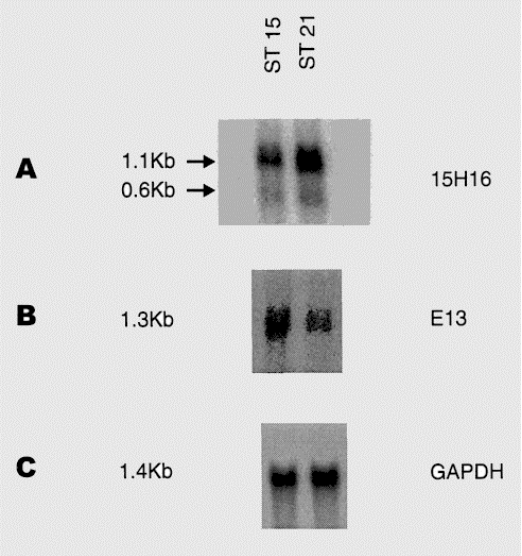


Figure 4 Northern blot analysis of chicken phospholamban and skeletal α-tropomyosin in stage 15 and stage 21 hearts. The phospholamban probe (15H16) detected two RNA bands of 1.1Kb and 0.6Kb with higher steady-state levels in the stage 21 sample (panel A). The skeletal α-tropomyosin probe (E13) hybridized to one single band with the message size of 1.3Kb (panel B). The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probe was used as a control to show the loading of RNA samples in each lane (panel C).

myocardium was gradually diminished and at the stage 21 embryo, only the atrial myocardium expressed the E13 gene (Fig 5D). By Northern blot analysis, the E13 probe detected a 1.3Kb message in the total RNA derived from both staged hearts (Fig 4B). Consistently, the steady-state level of E13 message was decreased from stage 15 heart to stage 21 heart. However, no change in the level of message of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected by Northern blot analysis of RNA derived from stage 15 and stage 21 hearts (Fig 4C). Therefore, skeletal α-tropomyosin recognized by the E13 probe was decreased in the developing heart temporally and spatially.

Expression of a novel 21C gene is increased at AV canal region of developing hearts

From the differential display and whole-mount *in situ* hybridization screening, one (21C) of the several novel genes isolated showed a cardiac-specific expression pattern during early chicken embryonic development. Whole-mount *in situ* hybridization using the 21C riboprobe revealed hybridization signals in the hearts of both stage 15 and stage 21 embryos(Fig 6A and 6B). Sectioning through these embryos after whole-mount *in situ*

hybridization revealed that the 21C gene expression was confined to myocardium (Fig 6C and 6D). At stage 15, a strong signal was found in myocardia of atrium and outflow tract (Fig 6C). As the development advanced to stage 21, a strong hybridization signal was detected by the 21C riboprobe in the ventricular myocardium (Fig 6B and section data not shown). The AV canal myocardium from stage 21 heart showed an increase in the expression of this 21C gene as compared to that from stage 15 heart. This is consistent with the isolation of the 21C clone from the stage 21 RNA sample using the differential display method.

To further examine the expression pattern of the 21C gene in early developing heart, we performed whole-mount *in situ* hybridization on stage 8^+ and stage 13 embryos. At stage 8^+, the 21C gene was exclusively expressed in the initially paired primordia located on either side of the embryonic midline (Fig 7A). Careful sectioning through the embryo demonstrated that 21C expression was restricted to the cardiac mesoderm and not found in any other tissues (Fig 7B). This cardiac-specific expression pattern was still detected in stage 13 embryo (Fig 7C and 7D) in which the heart tube had formed and looped to the right side of the embryo.

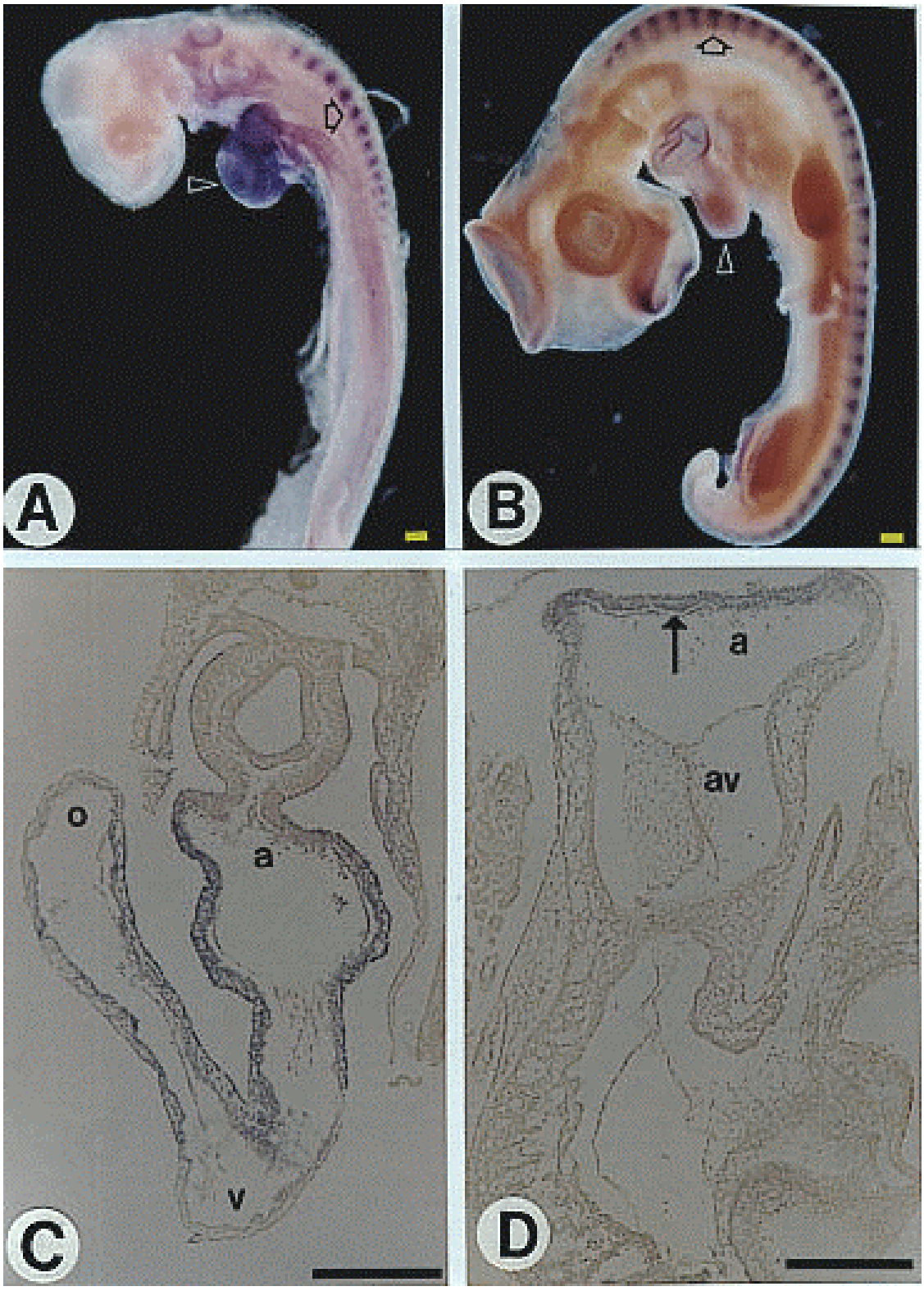
Northern blot analysis of total RNA isolated from stage 15 and stage 25 hearts suggested that the 21C gene encodes a single 9.5Kb message (Fig 8B). It is also clear that the steady-state level of the 21C expression in stage 25 heart is much higher than that in the stage 15 heart (Fig 8B). This result together with the *in situ* hybridization data suggest an increased expression in the heart as the development of embryos proceeded. However, our *in situ* data do not allow us to distinguish whether more cells at the AV regions of stage 21 heart express the 21C gene or each cell at this region expresses more 21C gene or both. In a preliminary experiment, we found that the 21C expression diminished in the day 12 embryonic heart (data not shown).

For all cloned cDNA fragments used for making anti-sense riboprobes, sense probes were also made and used as negative controls. One example is shown in Fig 9, where the 21C sense riboprobe was used. This probe did not hybridize to either stage 15 (Fig 9A) or stage 21 (Fig 9B) embryos. Same results were obtained from 15H16 and E13 sense probes (data not shown).

Figure 5 (Page 8) Expression patterns of E13 clone (chicken skeletal α-tropomyosin) in stage 15 and stage 21 embryos by *in situ* hybridization. (A and C) stage 15 embryo; (B and D) stage 21 embryo. (A and B) dark-field micrographs of whole embryos after *in situ* hybridization; (C and D) bright-field micrographs of cross sections through hearts of the same embryos in A and B, respectively. The E13 message was detected strongly in hearts (arrowheads) and somites (open arrows) of embryos at both stage 15 and stage 21 (A and B). Hybridization signals were drastically reduced in stage 21 heart as compared to that in stage 15 heart (A and B). Furthermore, in the cross section samples, the E13 gene was widely expressed in the entire myocardium of the stage 15 heart (C), whereas its expression level was decreased and restricted to the atrial myocardium of the stage 21 heart (arrow in D). There was no signal detected in the AV canal region of the stage 21 heart. a, atrium; av, atrioventricular canal; o, outflow tract; v, ventricle. Scale bar = 200μm.

Figure 6 (Page 9) Expression patterns of 21C clone (a novel, cardiac-specific and differentially expressed gene) in stage 15 and stage 21 chicken embryos by *in situ* hybridization. (A and C) stage 15 embryo; (B and D) stage 21 embryo. (A and B) dark-field micrographs of whole embryos after *in situ* hybridization; (C and D) bright-field micrographs of cross sections through hearts of the same embryos in A and B, respectively. Embryos were viewed from the right-side to show the ventricle region (v) and outflow tract (o) of the hearts. The 21C gene was expressed in the stage 15 heart with highest level in the myocardial cells of both atrium (a)and outflow tract (o), whereas the 21C gene expression in the stage 21 heart (D) was found in the myocardial cells of the outflow tract (o), ventricle, AV canal, and atrium (a), but not in the AV cushion (avc). Scale bar = 200μm.

Figure 7 (Page 10) Expression patterns of 21C clone in early stage chicken embryos visualized by *in situ* hybridization. (A) Ventral view of the stage 8^+ (6 somites) embryo after whole-mount *in situ* hybridization. The 21C gene expression was restricted to the paired thickened splanchnic mesoderm, the primordia of the heart (arrowheads). (B) Cross-section of the same embryo as shown in (A). The 21C gene expression was clearly detected in the cardiac mesoderm (arrowheads) but not in endoderm layers (en) which were just adjacent to the mesoderm tissues. (C) Whole-mount *in situ* hybridization of a stage 13 embryo. The 21C was strongly expressed in the heart (arrowhead). (D) Sagittal section of the same embryo as shown in the (C). The 21C expression was only detected in the myocardial cells (m) of the heart, but not in the other parts of the embryo. (A) dark-field micrograph, (B, C, and D) bright-field micrographs. Scale bar = 200μm.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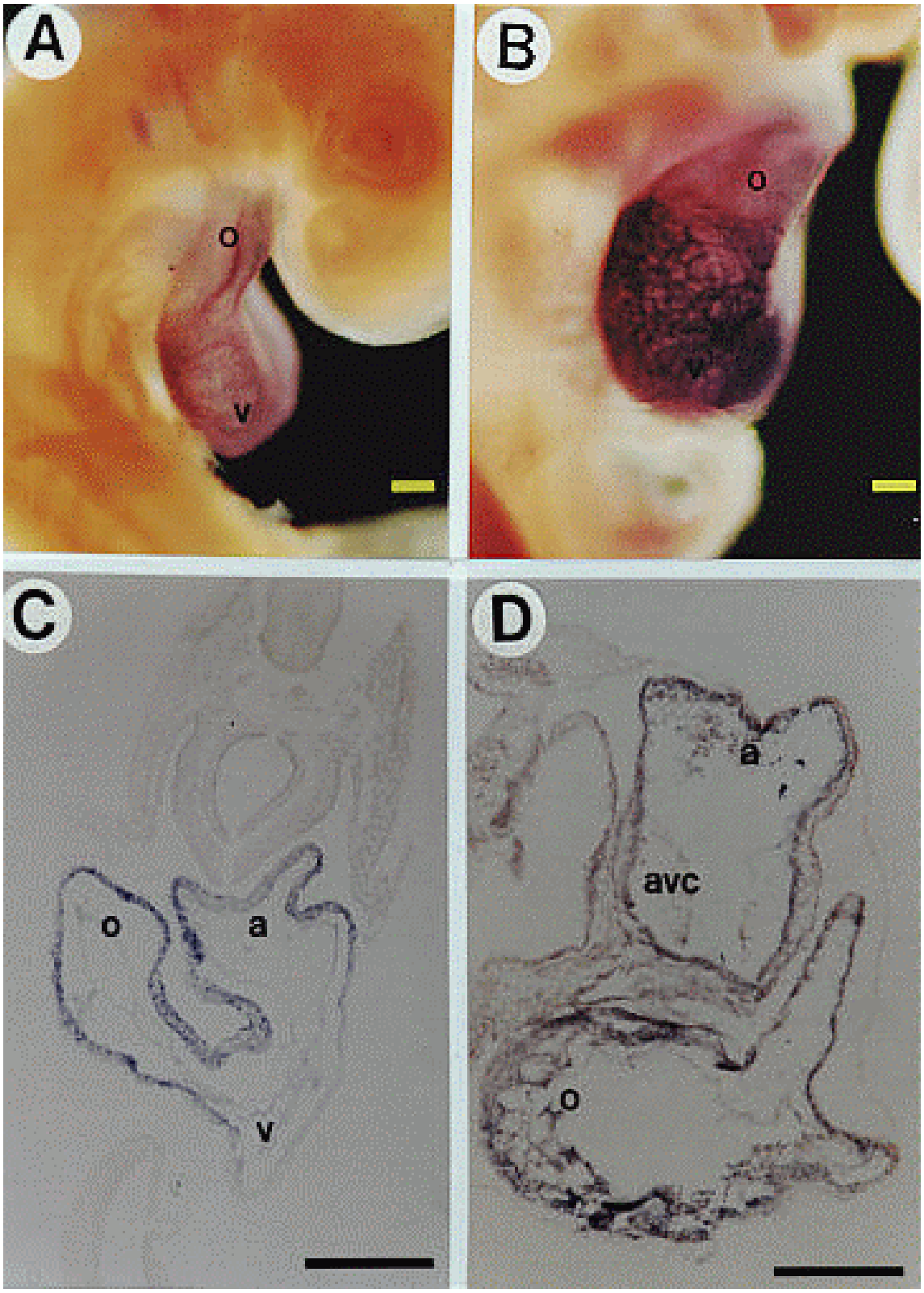
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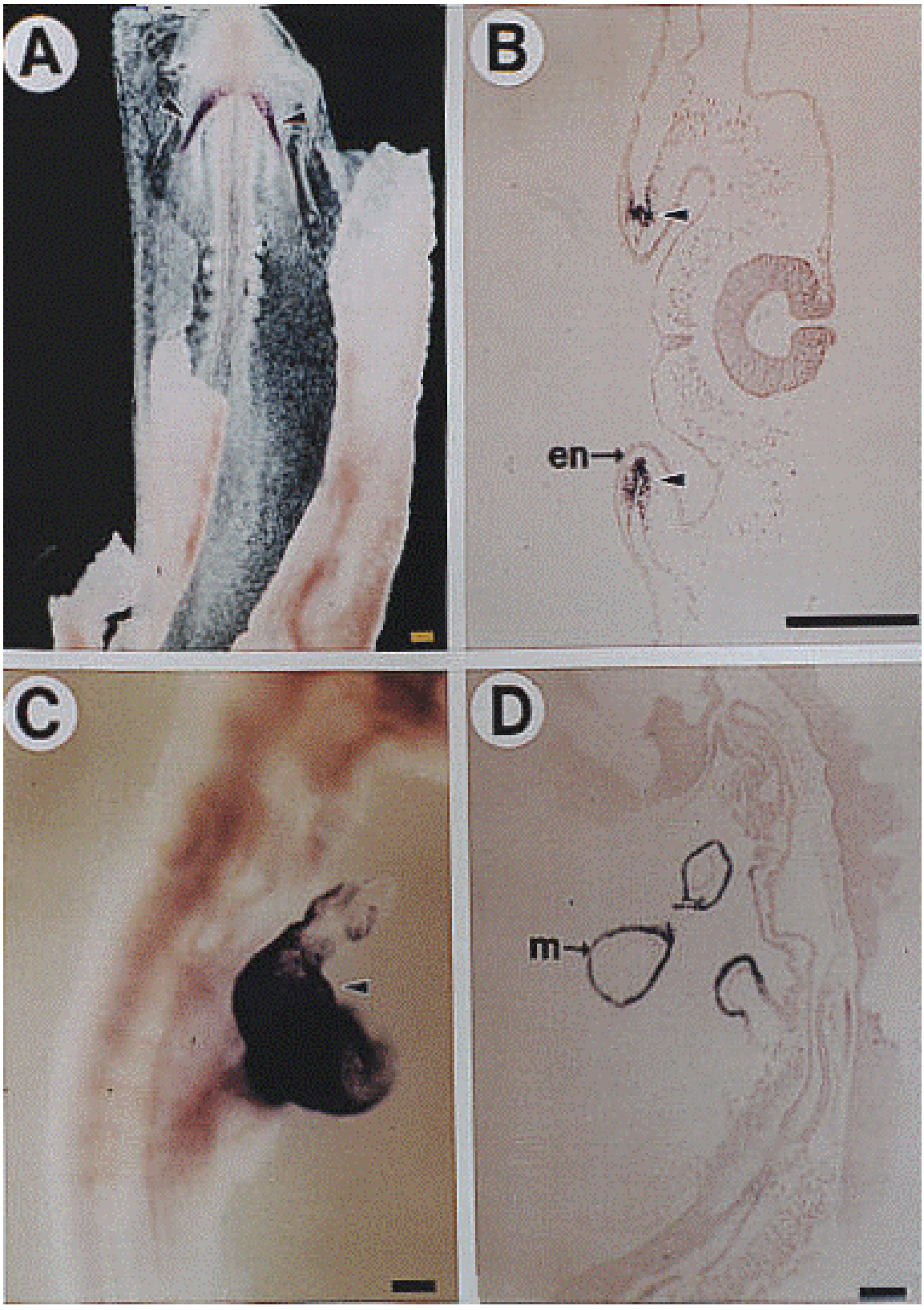
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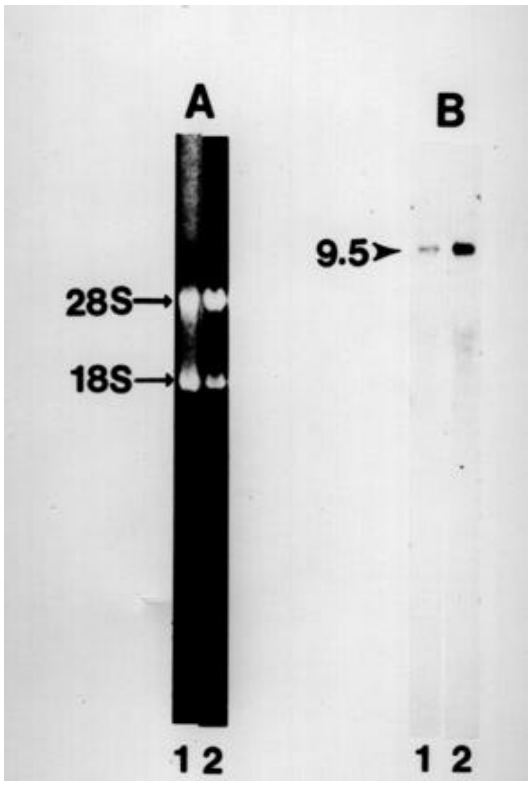

Figure 8 Northern blot analysis of 21C gene transcript in stage 15 and stage 25 chicken embryonic hearts. The cloned 21C fragment was used to probe total RNA (10 μg each) isolated from stage 15 (lane1) and stage 25 (lane 2) hearts. (A) shows the total RNA loading as visualized by ethidium bromide staining. (B) shows the Northern blot result with the 21C probe. Note that a 9.5 Kb band detected by the 21C probe was presented at both stage 15 (lane 1) and stage 25 (lane 2) . However, there was a significant increase in the steady-state level of the 21 C transcript in the stage 25 heart as compared to that of the stage 15 heart.

DISCUSSION

In this study, the differential mRNA display method was employed to identify and to clone differentially expressed genes, which may be important for the formation of AV valves and septa. A critical period in the valve and septum formation has been previously identified to be between stage 15 and stage 21 of chicken development (15). During this period, an epithelial-mesenchymal cell transformation appears to occur through the induction of certain endocardial cells in the AV canal region of developing heart to become mesenchymal cells by local signals produced from the AV myocardium (13-15). Therefore, we performed our differential display on the RNAs prepared from AV tissues of these two stages to specifically identity candidate genes involved in the valve and septum formation. As a potentially rapid and efficient method of screening differentially expressed genes, differential display has several technical advantages (11) as well as several problems (19). The amount of total RNA used in the differential display is 2-3μg and this amount of RNA, in combination with 10 different sets of primers, is enough to display at least 5% of the total mRNA represented. Moreover, there is no need to isolate poly(A) RNA in this approach. However, one of the obstacles is the rather high number of 'false positive' cDNA products from the primary screening that cannot be confirmed by Northern blot analysis. In this regard, in order to limit the positive clones to those that are cardiac-specific and are differentially expressed genes during this developmental period, we differentially displayed RNAs isolated from hearts and from whole embryos without hearts at both stages of development. Although the majority of clones obtained (Table 1) were not cardiac-specific, three examples given in this study did show differential expression in the AV canal regions of stage 15 and stage 21 hearts. Furthermore, whole-mount *in situ* hybridization was also employed in this study as a secondary screening method. This approach allowed us to quickly confirm the spatial and temporal expression patterns of isolated genes. In addition, the success rate (83%) of the whole-mount *in situ* hybridization in this study was much higher than that for Northern blot analysis (29%). Moreover, the Northern blots required a large amount of total RNA, which, in general, was difficult to obtain from the early developing embryos. In addition, the information regarding the spatial pattern of expression could not be obtained by the Northern blot analysis. Moreover, contradictory results were observed when data from the Northern blot analysis was compared with the differential display results. One such example was seen with the clone 15H16 (phospholamban). The phospholamban messages were significantly higher in the AV canal region of stage 15 heart than that of stage 21 heart (Fig 3). However, the Northern blot analysis of RNAs from stage 15 and stage 21 hearts showed an opposite result (Fig 4). Therefore, the whole-mount *in situ* hybridization is more useful for validation of the differential display data. In fact, in a recent report, whole-mount *in situ* hybridization was used to screen randomly sequenced cDNA clones to identify expression patterns of novel genes (33). The redundancy and under-representation of certain mRNA species in the differential display may represent another problem. However, it has been suggested that this problem may be overcome by using one-base anchored oligo-dT primers instead of two-base anchored oligo-dT primers (34).

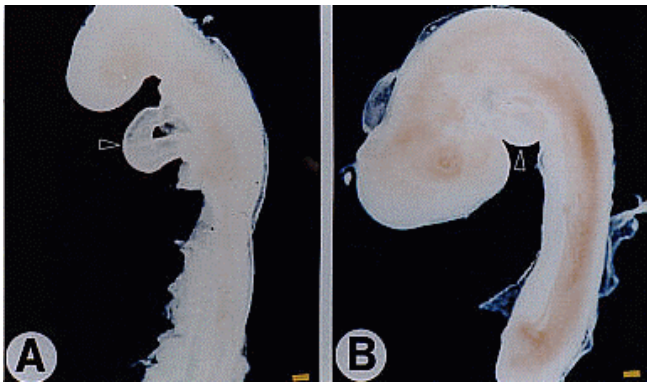

Figure 9 (Page 12) Whole-mount *in situ* hybridization of stage 15 and stage 21 chicken embryonic hearts using the sense riboprobe. The DIG-labeled 21C sense riboprobe was used as a negative control to probe stage 15 (A) and stage 21 (B) embryos. There was no detectable signal found in both staged embryos. Arrowheads point to the hearts. Scale bar = 200μm.

Table 1. Characteristics of cDNA fragments isolated from differential display of mRNAs prepared from the atrioventricular region of stage 15 and stage 21 chicken embryonic hearts.

Clone Name	Size(bp)	Sequence Homology	Whole-Mount *In Situ* Pattern
15A[a]	491	eIF-4AII[b]	ubiquitous[f]
15H1	240	none	heart, tail
15H16[a]	320	phospholamban[c]	heart
15H2	250	none	heart, brain
15H3	280	none	Not tested
21C[a]	251	none	heart
21G[a]	365	none	Undetectable
21H1	280	none	Undetectable
21H2	400	none	ubiquitous[f]
E10	380	none	heart, neural tube, limb
E13	268	α-tropomyosin[d]	heart, somite
H1	200	none	ubiquitous[f]
H2	200	α-actin[e]	Not tested
H3	400	none	ubiquitous[f]

a. These clones were generated by subcloning respective cDNA fragments into the pBK vector, whereas the rest of clones were done in the pCRII vector.
b. Mouse protein synthesis initiation factor eIF-4AII (38).
c. Chicken cardiac phospholamban (24).
d. Chicken skeletal α-tropomyosin (32).
e. Chicken cardiac α-actin (39).
f. Their messages were expressed in more than 5 different organs/tissues including heart. However, among these clones, there were different expression patterns observed in the whole-mount *in situ* hybridization.

The decrease in the phospholamban at the AV canal region of developing hearts between stage 15 and stage 21 may correlate to the drastic change from heart tubes to heart chambers. The heart tube at the stage 15 performs a peristaltic contraction throughout the whole tube. An evenly distributed phospholamban in the myocardium of tubular heart may be needed to control the sarcoplasmic reticulum Ca^{++} pump activity during myocardial contraction. When the valves and septa are partially formed at the stage 21, a rapid and regulated contraction is mainly carried out by the myocardia of atria and ventricles.

Therefore, an increase in phospholamban expression is needed in these areas of the heart. In contrast, the AV myocardium may not require this type of contraction and therefore, a decrease in expression of phospholamban occurrs. What mechanism/signal is responsible for the down-regulation of phospholamban gene in the AV myocardium between stage 15 and stage 21 remains to be determined. It is of interest to note that a down-regulation of N-CAM gene in the AV endocardium also occurs during this period of development and is believed to be required for the epithelial-mesenchymal cell transformation (14). One plausible mechanism is that the down-regulation of both phospholamban and N-CAM genes in the same AV canal region at this period of development may be a developmental response to a similar signal. In this regard, it should be of interest to examine whether a common regulatory element might exist in the promoters/enhancers of both genes.

The decrease in the expression of skeletal α-tropomyosin in hearts from stage 15 to stage 21 did not appear to be restricted to the AV myocardium. Thus, the mechanism/signal responsible for this decrease in skeletal α-tropomyosin may be different from that inducing the down-regulation of phospholamban in the AV myocardium during the same period of development. It has been previously shown that the decrease in skeletal α-tropomyosin synthesis in the developing heart was accompanied with an increase in cardiac-specific α-tropomyosin (29). This type of developmentally regulated isoform switching during heart development was also seen for many contractile and regulatory proteins (35-37), although the molecular mechanism(s) for isoform switches is not completely understood.

In this study, a novel, cardiac-specific and differentially expressed gene, 21C, was identified and cloned. In a preliminary screening of cDNA libraries with the 21C fragment probe, we have obtained several overlapping clones. The composite insert sequenced contains about 3Kb of sequence upstream from the poly (A) tail of the 21C transcript. Using larger cDNA insert as a probe for the whole-mount *in situ* hybridization, an identical temporal and spatial expression pattern as that shown in this study was obtained (data not shown). The expression of the 21C gene appears to start at very early stage and possibly diminishes during the later stages of embryonic heart development. This suggests that the 21C gene may play an important role in cardiac morphogenesis.

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REFERENCES

1. DeHaan RL. Morphogenesis of the vertebrate heart. In: DeHaan RL. Ursprung H. eds. *Organogenesis.* New York. NY: Holt Rinehart & Winston Inc. 1965: 377-419.

2. Fishman MC, and Stainier DYR. Cardiovascular Development—Prospects for a Genetic Approach. *Circ Res* 1994; 74:757-763.

3. Bisaha JG, and Bader D. Identification and characterization of a ventricular-specific myosin heavy chain, VMHC1: Expression in differentiating cardiac and skeletal muscle. *Dev Biol* 1991; 148: 355-364.

4. Yutzey KE, Rhee JT, and Bader D. Expression of the atrial-specific myosin heavy chain AMHC1 and the establishment of anterioposterior polarity in the developing chicken heart. *Development* 1994;120: 871-883.

5. Drysdale TA, Tonissen KF, Patterson KD, Crawford MJ, and Krieg PA. Cardiac troponin I is a heart-specific marker in the Xenopus embryo: Expression during abnormal heart morphogenesis. *Dev Biol* 1994; 165: 432-441.

6. Ruzicka DL, and Schwartz RJ. Sequential activation of α–actin genes during avian cardiogenesis: Vascular smooth muscle α–actin gene transcripts mark the onset of cardiomyocyte differentiation. *J Cell Biol* 1988; 107: 2575-2586.

7. Edmondson DG, Lyons GE, Martin JF, and Olson EN. Mef2 gene expression marks the cardiac and skeletal lineages during mouse embryogenesis. *Development* 1994; 120: 1251-1263.

8. Bodmer R. The gene tinman is required for specification of the heart and visceral muscles in Drosophila. *Development* 1993; 118: 719-729.

9. Lints TJ, Parsons LM, Hartley L, Lyons I, and Harvey RP. Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development* 1993; 119: 419-431.

10. Komuro I, and Izumo S. Csx: a murine homeobox-containing gene specifically expressed in the developing heart. *Proc Natl Acad Sci USA* 1993; 90: 8145-8149.

11. Liang P, and Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 1992; 257: 967-971.

12. Bolender DL, and Markwald RR. Epithelial-mesenchymal transformation in chick atrioventricular cushion morphogenesis. *Scanning Electron Microsc* 1979: 313-321.

13. Potts JD, and Runyan BR. Epithelial-mesenchymal cell transformation in the embryonic heart can be mediated, in part, by transforming growth factor . *Dev Biol* 1989; 134: 392-401.

14. Mjaatvedt CH, and Markwald RR. Induction of an epithelial-mesenchymal transition by an *in vivo* adheron-like complex. *Dev Biol* 1989; 136: 118-128.

15. Markwald RR, Rezaee M, Nadajima Y, Wunsch A, Isokawa K, Litke L, Krug E. Molecular basis for the segmental pattern of cardiac cushion mesenchyme formation: Role of ES/130 in the embryonic chick heart. In: Clark EB, Markwald RR, and Takao A, eds. *Developmental Mechanisms of Heart Disease.* Mt. Kisco, NY: Futura Press. 1995: 185-192.

16. Hamburger V, and Hamilton H. A series of normal stages in the development of the chick embryo. *J Morph* 1951; 88: 49-92.

17. Maniatis T, Fritsch EF, and Sambrook J, Eds. *Molecular Cloning*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1982:194-195.

18. Chomczynski P, and Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162: 156-159.

19. Liang P, Averboukh L, and Pardee AB. Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. *Nucleic Acids Res* 1993; 21: 3269-3275.

20. Marchuk D, Drumm M, Savlino A, and Collins F. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Res* 1991; 19: 1156.

21. Novy RE, Lin JL-C, and Lin JJ-C. Characterization of cDNA clones encoding a human fibroblast caldesmon isoform and analysis of caldesmon expression in normal and transformed cells. *J Biol Chem* 1991; 266: 16917-16924.

22. Hemmati-Brivanlou A, Frank D, Bolce ME, Brown BD, Sive HL, and Harland RM. Localization of specific mRNAs in Xenopus embryos by whole-mount *in situ* hybridization. *Development* 1990; 110: 325-330.

23. Riddle RD, Johnson RL, Laufer E, and Tabin C. Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 1993; 57: 1401-1416.

24. Toyofuku T, and Zak R. Characterization of cDNA and genomic sequences encoding a chicken phospholamban. *J Biol Chem* 1991; 266: 5375-5383.

25. Tada M, and Katz AM. Phosphorylation of the sarcoplasmic reticulum and sarcolemma. *Ann Rev Physiol* 1982; 44: 401-423.

26. Toyofuku T, Doyle DD, Zak R, and Kordylewski L. Expression of phospholamban mRNA during early avian muscle morphogenesis is distinct from that of α–actin. *Dev Dynamics* 1993; 196: 103-113.

27. Moorman AFM, Vermeulen JLM, Kohan MU, Schwartz K, Lames WH, and Boheler KR. Patterns of expression of sarcoplasmic reticulum $Ca2^{+}$-ATPase and phospholamban mRNAs during rat heart development. *Circ Res* 1995; 76: 616-625.

28. Montarras D, Fiszman MY, and Gros F. Characterization of the tropomyosin present in various chick embryo muscle types and in muscle cells differentiated *in vitro*. *J Biol Chem* 1981; 256: 4081-4086.

29.Wang S-M, Wang S-H, Lin JL-C, and Lin JJ-C. Striated muscle tropomyosin-enriched microfilaments of developing muscles of chicken embryos. *J Muscle Res Cell Motil* 1990; 11: 191-202.

30. Lindquester GJ, Flach JE, Fleenor DE, Hickman KH, and Devlin RB. Avian tropomyosin gene expression. *Nucleic Acids Res* 1989; 17: 2099-2118.

31. Forry-Schaudies S, Gruber CE, and Hughes SH. Chicken cardiac tropomyosin and a low-molecular-weight nonmuscle tropomyosin are related by alternative splicing. *Cell Growth & Differ* 1990; 1: 473-481.

32. Lemonnier M, Balvay L, Mouly V, Libri D, and Fiszman MY. The chicken gene encoding the (isoform of tropomyosin of fast-switch muscle fibers: Organization, expression and identification of the major proteins synthesized. *Gene* 1991; 107: 229-240.

33. Bettenhausen B, and Gossler A. Efficient isolation of novel mouse genes differentially expressed in early postimplantation embryos. *Genomics* 1995; 28: 436-441.

34. Liang P, Zhu W, Zhang X, Guo Z, O'Connell RP, Averboukh L, Wang F, and Pardee AB. Differential display using one-base anchored oligo-dT primers. *Nucleic Acids Res* 1994; 22: 5763-5764.

35. Caplan AI, Fiszman MY, and Eppenberger HM. Molecular and cell isoforms during development. *Science* 1983; 221: 921-927.

36. Swynghedauw B. Developmental and functional adaptation of contractile proteins in cardiac and skeletal muscles. *Physiol Rev* 1986; 66: 710-770.

37. Jin JP, and Lin JJ-C. Isolation and characterization of cDNA clones encoding embryonic and adult isoforms of rat cardiac troponin T. *J Biol Chem* 1989; 264: 14471-14477.

38. Nielsen PJ, and Trachsel H. The mouse protein synthesis initiation factor 4A gene family includes two related functional genes which are differentially expressed. *EMBO J* 1988; 7: 2097-2105.

39.Eldridge J, Zehner Z, and Paterson BM. Nucleotide sequence of the chicken cardiac alpha actin gene: Absence of strong homologies in the promoter and 3'-untranslated regions with the skeletal alpha actin sequence. *Gene* 1985; 36: 55-63.