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Forced expression of the homeodomain protein Gax inhibits cardiomyocyte proliferation and perturbs heart morphogenesis

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SUMMARY

The development of the tubular heart into a complex four-chambered organ requires precise temporal and region-specific regulation of cell proliferation, migration, death and differentiation. While the regulatory mechanisms in heart morphogenesis are not well understood, increasing attention has focused on the homeodomain proteins, which are generally linked to morphogenetic processes. The homeodomain containing gene *Gax* has been shown to be expressed in heart and smooth muscle tissues. In this study, the Gax protein was detected in the nuclei of myocardial cells relatively late in chicken heart development, at a time when myocyte proliferation is declining. To test the hypothesis that the Gax protein functions as a negative regulator of cardiomyocyte proliferation, a replication-defective adenovirus was used to force its precocious nuclear expression during chicken heart morphogenesis. In experiments in which Gax- and β-galactosidase-expressing adenoviruses were co-injected, clonal expansion of myocytes was reduced, consistent with inhibition of myocyte proliferation. This effect on proliferation was corroborated by the finding that the percentage of exogenous Gax-expressing myocytes that were positive for the cell cycle marker PCNA decreased over time and was lower than in control myocytes. The precocious nuclear expression of Gax in tubular hearts resulted in abnormal heart morphology, including small ventricles with rounded apices, a thinned compact zone and coarse trabeculae. These results suggest a role for the Gax protein in heart morphogenesis causing proliferating cardiomyocytes to withdraw from the cell cycle, thus influencing the size and shape that the heart ultimately attains.

Abbreviations: Gax, Growth Arrest Homeobox; PCNA, proliferating cell nuclear antigen; β-gal, β-galactosidase; CMV, cytomegalovirus; RSV, Rous sarcoma virus; bHLH, basic helix-loop-helix; MEF-2, myocyte enhancer factor-2

Key words: heart development, cardiogenesis, homeodomain, protein, Gax, cardiomyocyte, chick

INTRODUCTION

The period during which the heart develops from a simple tube into a four-chambered organ is characterized by precise coordination of cell behavior including stage- and region-specific regulation of cell proliferation, migration, death and differentiation. The positional fate of the myocardial cells within the complex heart structure appears to be determined long before the tube has formed, when the cells exist as undifferentiated aggregates in the lateral mesoderm (Lee et al., 1994; Rosenquist, 1970, 1985; Garcia-Martinez and Schoenwolf, 1993; Litvin et al., 1992). The molecular mechanisms used by these primitive cardiac cells to drive the cascade of events that eventually results in the complex structure of the mature vertebrate heart are beginning to be studied but are not well understood.

Homeodomain genes, first identified as determinants of the body plan in *Drosophila* (Lawrence and Morata, 1994), are good candidates for being morphogenetic determinants in vertebrate tissues (Krumlauf, 1994). A number of vertebrate homeodomain genes have now been identified, a significant subset of which are expressed in the developing heart (Leussink et al., 1995; Chan-Thomas et al., 1993; Pexieder, 1995; Bodmer, 1995). The Nkx family of homeodomain proteins is one of the earliest markers of the cardiomyocyte lineage (Lee et al., 1996; Schultheiss et al., 1995; Evans et al., 1995; Tonissen et al., 1994; Lints et al., 1993; Bodmer, 1993) and the disruption of *mNkx2.5* gene results in the arrest of cardiac morphogenesis at an early straight tube stage (Lyons et al., 1995). Targeted disruption and antisense experiments have also identified other transcription factors that are necessary for normal heart development (Sucov et al., 1994; Chen et al., 1994; Srivastava et al., 1995), but there is still little understanding of the regulatory mechanisms by which the pre-

cardiac mesoderm develops the complex structures of the mature heart of constant size, shape and cell number.

This study utilized a novel, adenoviral-mediated approach to study the molecular mechanisms of heart morphogenesis. After identifying the pattern of expression of the endogenous homeodomain-containing Gax protein, precocious expression was forced in vivo by delivery of an adenoviral vector into the developing chick heart. The consequences of forced Gax protein expression support a role for Gax as a negative regulator of cardiomyocyte proliferation. In this role, Gax may ultimately determine the shape and size that the developing heart attains.

MATERIALS AND METHODS

Immunohistochemical analysis of Gax protein expression

Chick embryos (*Gallus gallus*) were harvested at representative stages between 13 and 41 (Hamburger and Hamilton, 1951), fixed in 10% formalin-acetate-buffered solution (Ted Pella, Inc., Redding CA) and processed for frozen sectioning (Watanabe et al., 1992). Serial 20 μm sections in a plane frontal to the heart were collected and stained for Gax protein using the indirect antibody technique with polyclonal anti-Gax raised against the N terminus of the Gax protein, which is conserved across species (Candia et al., 1993). This antibody specifically recognizes the Gax protein on western blotting (not shown). For single antibody staining, the second antibody directed to the appropriate immunoglobulin subtype was coupled to FITC (Cappel). For the double-labeled sections, the anti-Gax binding was detected using goat anti-rabbit IgG (H and L, human and mouse IgG adsorbed) conjugated to Texas Red (Southern Biotech. Assoc. #4050-07). The mf20 monoclonal antibody against light meromyosin (labels skeletal and cardiac but not smooth muscle myosin (Bader et al., 1982)) was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD. The mf20 binding was detected using an anti-mouse IgG (Fc) conjugated to FITC (Cappel #55662). Negative controls for the double-labeling experiment were treated with the individual first antibodies, anti-Gax or mf20, followed by the inappropriate second antibody. Negative controls exhibited no immunostaining above levels observed in negative control sections where the first antibodies were eliminated altogether. Observations were made and photographs taken at the light microscope level with the appropriate filters for FITC and RITC using conventional fluorescence microscopy (Nikon, Diaphot200) or laser confocal microscopy (Zeiss LSM 410; Zeiss ×40 Plan Neofluor, 0.90 NA, oil immersion). Images were collected in 0.5 μm sections and shown as a collapsed image of 14 sections.

Replication-defective adenoviral constructs

Replication-defective recombinant adenoviral constructs expressing Gax or β-galactosidase (β-gal) under the control of either cytomegalovirus (CMV) or Rous sarcoma virus (RSV) promoters were generated, and are referred to as AdCGNGax, AdRSVGax and AdRSVGal. The construction of AdCGNGax is described elsewhere (Smith et al., 1997) and contains the rat Gax cDNA fused to the N-terminal influenza virus hemagglutin (HA) epitope (J. Field). Upstream is the CMV early promoter linked to the *tk* 5′ UTR, and downstream is the rabbit globin 3′ UTR containing splicing and polyadenylation signals. The construction of AdRSVGal (Stratford-Perricaudet et al., 1992) and AdRSVGax have been previously described, and are E1-deleted recombinant adenoviruses expressing β-gal with a nuclear localization signal, or expressing Gax, under the control of the RSV long terminal repeat promoter. The AdCGNGax construct was utilized so that expression of exogenous Gax could be easily detected and distinguished from the endogenous protein using a commercially available (anti-HA) antibody. The AdRSV Gax construct was used to produce expression of the exogenous protein uniformly throughout the heart (Fisher and Watanabe, 1996). Growth and purification steps of recombinant adenovirus were as described (Smith et al., 1997). In brief, replication-defective recombinant adenovirus were co-transfected in 293 cells with the large *Cla*I fragment of the AD5 d1324 viral DNA, amplified through subculture, purified with two CsCl gradient centrifugations, titered by 293 plaque assay (expressed as plaque forming units (pfu) per ml), and stored at –80°C in 10% glycerol.

Application of recombinant adenovirus to embryonic chicken hearts

Recombinant adenovirus at titers of 10^{10}-10^{11} pfu/ml were applied to the surface of stage 13-16 hearts in ovo as previously described (Fisher and Watanabe, 1996). In brief, fertile chicken eggs after 64-68 hours of incubation (stage 13-16, early heart looping) were drained into a shallow well of Saran Wrap™ within a plastic cup. The vitelline membrane surrounding the beating heart was incised to expose the surface of the heart. Approximately 500 nl (5×10^6 to 5×10^7 pfu) of adenovirus solution was injected via a 30 μm bore micropipette around the heart under stereoscopic guidance. The embryos were incubated in humidified air at 37.5°C for up to an additional 96 hours prior to analysis. The application of AdRSVGal was used as the control for the application of AdRSVGax and AdCGNGax. Control and experimental viruses were applied in similar concentrations and volumes, and in the same vehicle. Our previous studies have demonstrated that this method of application of recombinant adenovirus (AdRSVGal) does not perturb cardiac morphogenesis and that expression of exogenous protein is limited to cardiomyocytes (Fisher and Watanabe, 1996).

Cell proliferation assays

To measure the effect of forced expression of Gax on cell proliferation, two different assays were used. The first assay was a clonal analysis, as previously described (Fisher and Watanabe, 1996), modified for the purposes of this study. A 1:100 dilution of AdRSVGal was mixed with an equal volume of undiluted AdRSVGax and applied to the heart surface. At 24 and 72 hours after application, embryos were fixed and stained for β-gal, and the number of nuclei per clone counted by stereomicroscopic examination. Embryo hearts with 20-50 clones were counted, with 4-6 embryos per group. These clonal assays were performed on ventricular tissues only, as not enough clones were observed in the atria or outflow tracts to perform an analysis. A possible confounding factor of competition between the two viral constructs was excluded by assays in which the amount of AdRSVGal injected was held constant, and AdRSVGax or AdCGNGax was co-infected at ratios from 1:100 to 1:1. Quantitation of β-gal activity demonstrated no difference with single or double adenovirus infections (data not shown).

The second assay utilized PCNA as a marker for proliferative cells (Kurki et al., 1986). Embryos were harvested, fixed and sectioned as described above. Immunostaining was performed using a mouse monoclonal antibody against PCNA (Sigma, P-8825) at a dilution of 1:500 and rhodamine-conjugated goat IgG (Capell) as secondary antibody (1:1000) followed by Hoechst 33258 stain at 100 ng/ml for 10 minutes. Co-localization of exogenous Gax was achieved with an antibody to the hemagglutinin (HA) epitope directly tagged with fluorescein (Boehringer Mannheim, # 1666878) at a concentration of 10 mg/ml. Control samples (AdRSVGal treated) were handled in an identical manner. In selected samples, a monoclonal antibody to titin (Sigma, T-9030) or myosin was used to identify and distinguish myocardial cells.

Control and experimental slides were matched by anatomical

region, period of incubation and developmental stage. Areas demonstrating exogenous Gax expression (based on HA immunofluorescence) were identified. These sections were photographed (Ektachrome 400) using appropriate filters at ×400 or ×630 for PCNA staining, exogenous Gax staining and nuclear staining. These identical overlapping images were then used to quantify the number of exogenous Gax-positive cells (FITC, green), PCNA-positive cells (RITC, red), or cells positive for both (yellow) as a percentage of total nuclei (Hoechst, blue) within the compact myocardium of any given field. Control specimens were photographed in an identical fashion in corresponding anatomic regions. Eight microscopic fields were counted per embryo and three embryos per condition at each time point, with 700 cells per embryo at 48 hours and 72 hours, and 300 cells per embryo at 24 hours. Differences between the groups were assessed using an unpaired *t*-test, with $P<0.05$ determining statistical significance.

Examination of heart morphology

At 48-96 hours postinjection, embryos were fixed in a solution of 2% formaldehyde and 0.2% glutaraldehyde in PBS and photographed as whole mounts on a stereomicroscope (Nikon SMZ-2T with N6006 camera). The hearts were dissected from the embryos and photographed side-by-side with stage-matched controls (AdRSVGal-treated or no virus application). The limb and head morphologies were used as the distinguishing features of these stages (Hamburger and Hamilton, 1951). Intact embryonic hearts or hearts sliced open along a dorsoventral plane using a scalpel to reveal the ventricular chambers were also prepared for scanning electron microscopy (Hiruma and Hirakow, 1989). Whenever possible both halves of the hearts were prepared and observed. In brief, hearts dissected in PBS were immersed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.08 M cacodylate buffer at pH 7.6 for 2-12 hours at 4°C, postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer pH 7.6 for 1-4 hours at 4°C, dehydrated in increasing concentrations of ethanol, critical-point dried in liquid CO_2, coated with platinum and observed at 20 kV on a JEOL scanning electron microscope (JSM840A). Photographs of the experimental samples and their matched controls were taken at the same magnification using Type 55 Polaroid film.

RESULTS

Endogenous Gax expression in the developing chick heart

Endogenous Gax expression was examined in the developing chicken heart using previous observations in mouse heart development (Skopicki et al., 1997) to focus the analysis. Gax protein was not detected by immunohistochemistry in stage 20 to 30 chicken hearts (Fig. 1), when myocytes are highly proliferative, with the exception of a few nuclei present in a region of the inner curvature at the atrioventricular junction in stage 24-25 chicken hearts. By stage 33, a few anti-Gax-stained nuclei were present within the myocardium of the ventricle immediately adjacent to the epicardium. In contrast, a large number of Gax-positive nuclei were scattered throughout the heart myocardium at stages 35-41 (Figs 1, 2). The laser confocal microscopy technique (Fig. 2A) revealed that the Gax-positive nuclei in the myocardium included many rounded or oval nuclei immediately surrounded by mf20-stained cytoplasmic components, i.e. sarcomeric myosin. These nuclei most likely belong to cardiomyocytes. In addition, a number of Gax-positive nuclei within the myocardium were present in regions that were not stained with mf20. The flattened shape and linear pattern of alignment of some of these nuclei suggest that they may belong to cells of the vasculature. No Gax-positive staining was observed in the aorta at stage 39 but small numbers of nuclei were positive by stage 41. In summary, no appreciable level of Gax protein expression was detected in the myocardium until after stage 33, at a time when many of the specialized structures of the heart have already formed.

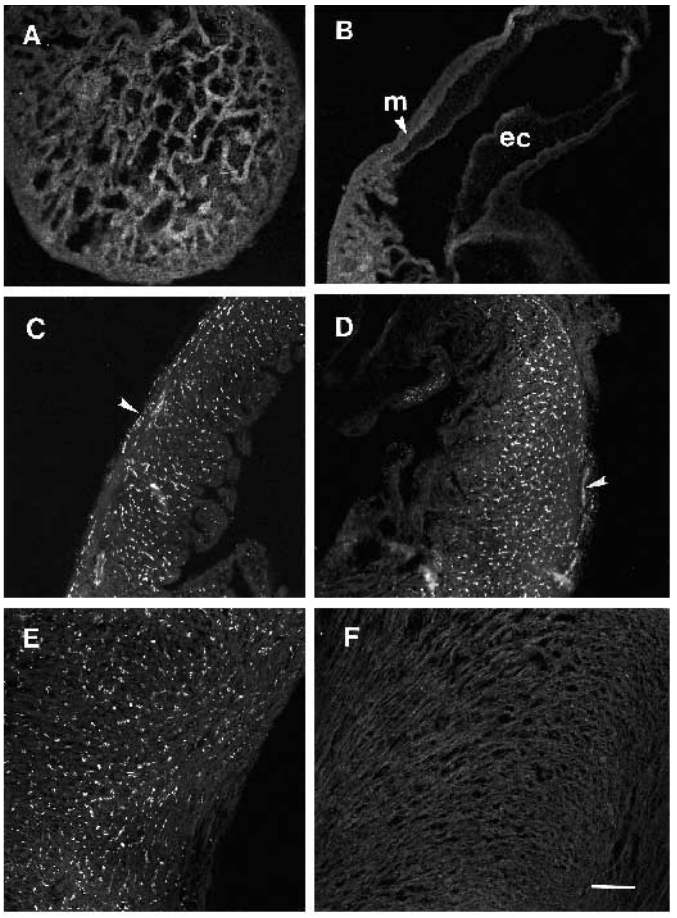

Fig. 1. Endogenous Gax expression. Gax-positive nuclei were not present in the stage 24 (A) ventricular or (B) outflow tract myocardium (m, myocardium; ec, endocardium) except in a focal region near the atrioventricular junction (not shown). In contrast, many Gax-positive nuclei were present by stage 39 (C-E) throughout the myocardium. Positive nuclei were abundant in the (C) right and (D) left ventricular wall and the (E) interventricular septum. Note staining of nuclei within the epicardium (arrowhead) in D. Control sections (F) with no anti-Gax antiserum added were negative. Bar, 100 μm for all panels.

Detection of adenovirally encoded Gax expression in chick heart

To test the function of the Gax protein during heart development, its expression was precociously forced in cardiomyocytes during heart morphogenesis in vivo. This was accomplished using replication-defective recombinant adenoviruses in an experimental system that we have previously characterized (Fisher and Watanabe, 1996). Recombinant adenovirus (5×10^6-5×10^7 pfu) expressing Gax under the control of a viral promoter was applied to the external surface of tubular hearts (stages 13-17) in ovo and the embryos

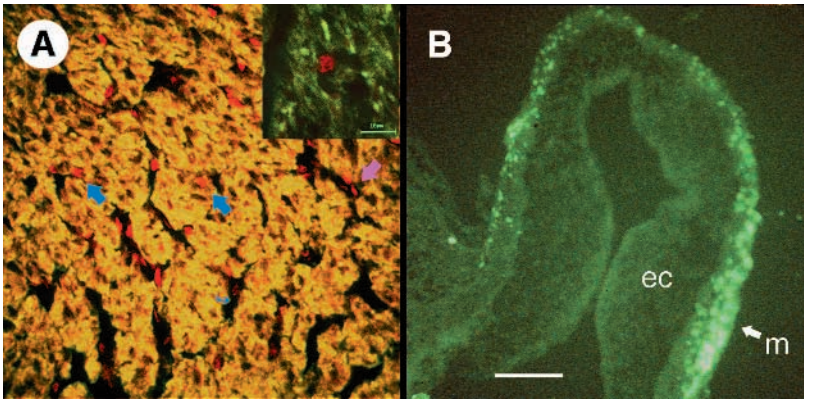

Fig. 2. Endogenous and exogenous Gax expression in the myocardium. (A) Laser confocal microscopy projected image of the stage 35 ventricular wall myocardium stained for endogenous Gax protein. Anti-Gax-positive oval and round nuclei (RITC, red nuclei indicated with blue arrowheads) are present within a meshwork of cardiac myosin tagged with mf20 (FITC, yellow). Gax-positive flattened nuclei are also present in regions not stained for myosin (purple arrowhead). The inset shows a higher magnification confocal image (1.2 μm step) of a cell that is co-stained for Gax and myosin.
(B) Exogenous Gax in stage 25 outflow tract myocardium infected with AdCGNGax. A high proportion of nuclei in the myocardium (m) were positive for nuclear localized Gax (anti-HA-FITC, yellow). Cells in the region of the endocardium (ec) are negative. Bar, 25 μm (A) and 100 μm (B).

incubated for several days. Utilizing antibodies to an HA epitope on the expressed Gax protein, nuclear expression of the exogenous Gax was evident as early as 12 hours after the application of the virus (not shown) and was maintained for at least 72 hours after adenovirus application (Fig. 2B). This timetable of expression was similar to that previously observed for the marker enzyme β-galactosidase (Fisher and Watanabe, 1996) and suggests that forced expression of Gax was not inducing cell death over this period. The exogenous Gax protein was detected only in the nuclei, and was present at a time when little or no endogenous nuclear Gax was present. The cells expressing the exogenous Gax were differentiated myocytes as evidenced by the co-immunolocalization of muscle-specific proteins myosin and titin.

Exogenous Gax expression affects myocyte proliferation

Two observations led us to examine the role of Gax in regulating the embryonic myocyte cell cycle. First, the appearance of Gax in the cardiomyocyte nucleus (and aortic smooth muscle cell nuclei) occurs relatively late in development, long after the myogenic gene program has been activated (Chien et al., 1993), at a time when proliferation declines (Mikawa et al., 1992; Jeter and Cameron, 1971). Second, its expression in vitro is up-regulated in smooth muscle cells co-incident with growth arrest (Yamashita et al., 1997; Gorski et al., 1993). These observations suggested the hypothesis that Gax might serve as a negative regulator of myocyte proliferation in the developing heart. To test this hypothesis, the effect of forced precocious nuclear expression of Gax on myocyte proliferation was examined using two assays, one of which measured clonal expansion after adenoviral infection. In previously published experiments (Fisher and Watanabe, 1996), we demonstrated that the application of a reduced titer (approximately 10^5 pfu) of AdRSVGal to the embryonic heart enabled the identification of the expansion of single infected myocytes to 2, 4 and greater than 4 cell clones at 24 to 96 hours after infection (Fig. 3). In the second assay, PCNA was used as a biochemical marker to identify cycling cells (Kurki et al., 1986).

In the first assay, AdRSVGax was co-injected at a ratio of 100:1 with AdRSVGal, which served as a clonal marker. In the ventricles, the number of nuclei per clone were counted at 24 and 72 hours postinfection (Fig. 4). In the control group, in which AdRSVGal alone was injected, the number of cells per clone increased from 1 and 2 at 24 hours to 4 or >4 at 72 hours postinfection, consistent with an approximate myocyte doubling time of 24 hours, as reported by others for this stage of heart development (Jeter and Cameron, 1971; Mikawa et al., 1992). When AdRSVGax was co-infected with AdRSVGal, the percentage of 1- and 2-cell clones at 24 hours was similar to controls (P>0.05 by chi squared analysis). By 72 hours postinjection, a significant reduction in the percentage of clones that were 4 or >4 cells in size, with persistence of the

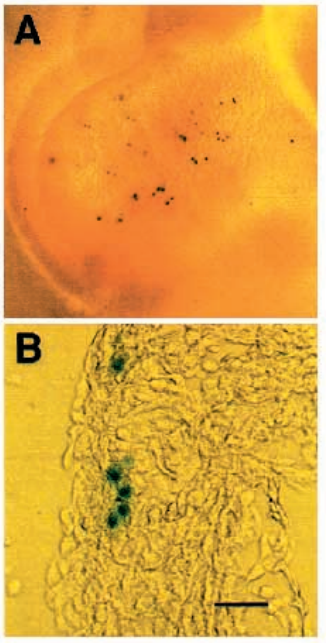

Fig. 3. Clonal analysis. Whole mounts of chicken embryo hearts stained for β-gal to assay for clonal expansion of myocytes. Stage 13-16 embryo hearts were exposed to low titer (10^5 pfu) AdRSVGal and incubated prior to analysis. At this dose of virus, the clusters of stained nuclei were well separated and could be distinguished from each other. 1- and 2-cell clones (A) can be detected on the left ventricular surface of whole mounts of chicken heart (2 mm wide) 24 hours after virus application. (B) 1- and 4-cell clones are evident in a frozen section of ventricular myocardium 48 hours after virus application. Bar, 40 μm.

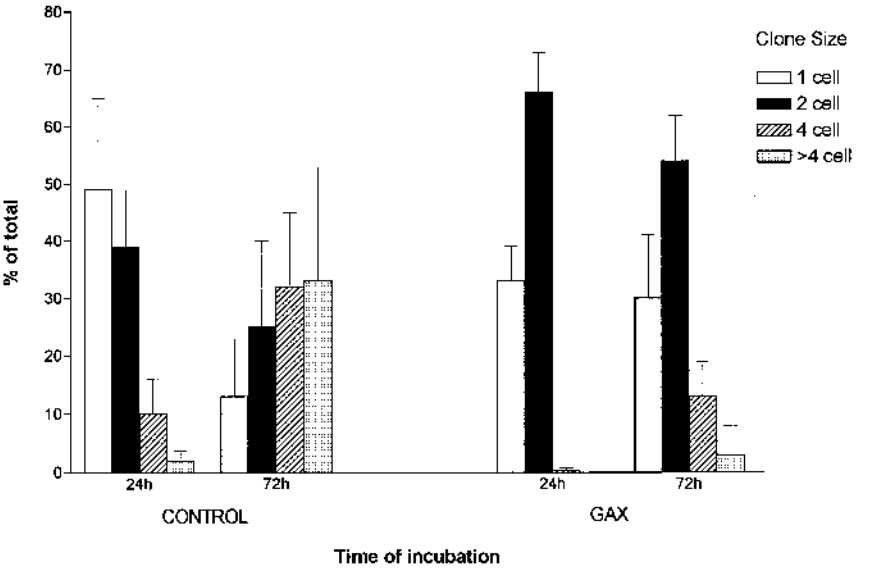

Fig. 4. Exogenous Gax inhibits clonal expansion. A 1:100 dilution of AdRSVGal alone (control) or mixed with an equal volume of undiluted AdRSVGax was applied to the heart surface. At 24 and 72 hours after application, embryos were fixed and stained for β-gal, and the number of blue nuclei (β-gal positive) per clone counted by stereomicroscopic examination. In the control hearts (AdRSVGal alone), the number of cells per clone increases with incubation time, consistent with an approximate cell doubling time of 24 hours. In experiments in which AdRSVGax was co-infected, inhibition of expansion to 4 and >4 cell clones was observed at 72 hours. *n*=4-6 embryos in each group, 20-50 clones per heart. Bar = s.d.

2-cell clones, was observed in the experimental group (P<0.001 by chi squared analysis). Thus, the number of cells carrying the β-gal marker is reduced in experiments in which the AdRSVGax expression vector is co-infected with the marker AdRSVGal virus.

To determine if the reduced cell number was due to an effect on the proliferation of the myocytes, the expression of proliferating cell nuclear antigen (PCNA), a subunit of DNA polymerase and marker for cycling cells (Kurki et al., 1986), was examined. Hearts infected with either AdCGNGax (experimental) or AdRSVGal (control) were co-stained for PCNA, the HA epitope to identify cells expressing exogenous Gax, and the Hoechst reagent to delineate all nuclei (Fig. 5A). The percentage of cells that were PCNA positive was similar in control (AdRSVGal; *n*=2, 300 cells) and AdCGNGax (*n*=2, 600 cells) -infected hearts 24 hours after application of virus (Fig. 5B). Subsequently, the percentage of myocytes which were PCNA positive in the control hearts was maintained between 40% and 48% (*n*=4, 650 cells). In the AdCGNGax-infected hearts, this percentage declined to 30% at 48 hours (*n*=2, 1500 cells) and 19% at 72 hours (*n*=3, 2000 cells) after viral application. When analysis was restricted to exogenous Gax-expressing cells, i.e. HA positive, the % that were PCNA positive declined from 41% at 24 hours to 28% at 48 hours and 14% at 72 hours after virus application (Fig. 5B). Thus, quantitation of both clonal cell numbers and expression of a biochemical marker of cell proliferation support the hypothesis that the forced precocious nuclear expression of the Gax protein inhibits embryonic cardiomyocyte proliferation.

To exclude the possibility that the effects of adenoviral-mediated forced expression of Gax were due to non-specific squelching of transcription, a second set of control experiments were performed in which another transcription factor was expressed under the same experimental conditions. p53 was selected for this purpose as the wild-type protein functions as a transcriptional repressor, but the protein is not required for

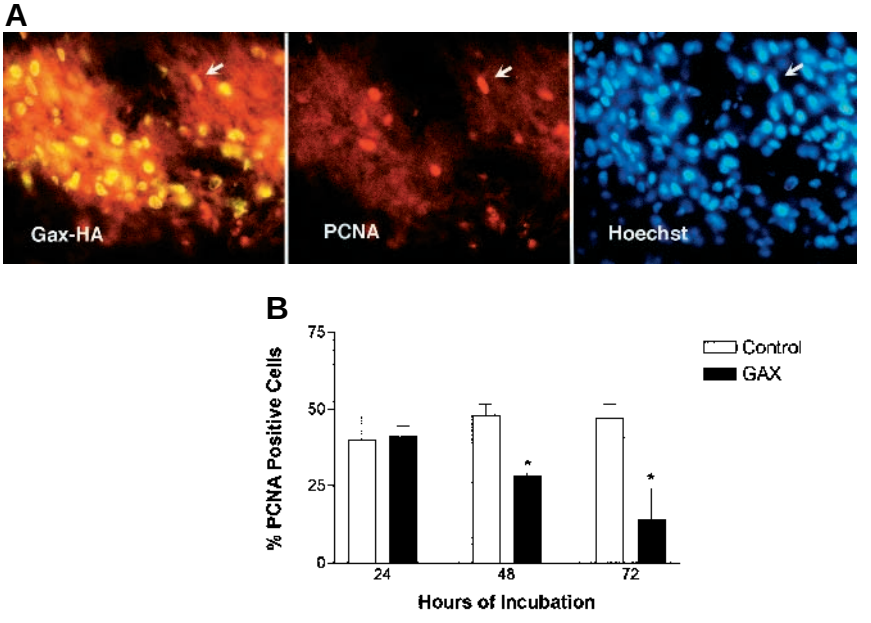

Fig. 5. PCNA and HA-tagged Gax co-staining of myocardial cells. (A) Heart tissue triple-stained for exogenous Gax-HA (FITC, yellow), proliferating cells using anti-PCNA (PCNA, RITC, red) and total nuclei with Hoechst dye (Hoechst, blue). Micrographs of the same field of sectioned myocardial tissue 72 hours after AdCGNGax application, photographed through three different filters. Nuclei expressing both Gax-HA and PCNA appear yellow-orange. A small proportion of cells are PCNA-positive and Gax positive (arrow). (B) The number of exogenous Gax-positive cells (FITC, green), PCNA-positive cells or cells positive for both (yellow) were determined as a percentage of total nuclei (Hoechst, blue) within the compact myocardium of sections as shown in (A). The % of nuclei that were PCNA positive are compared in control (AdRSVGal, white bars) and experimental (AdCGNGax, black bars) hearts. Bars = s.d. * indicates statistically significant differences (P<0.05).

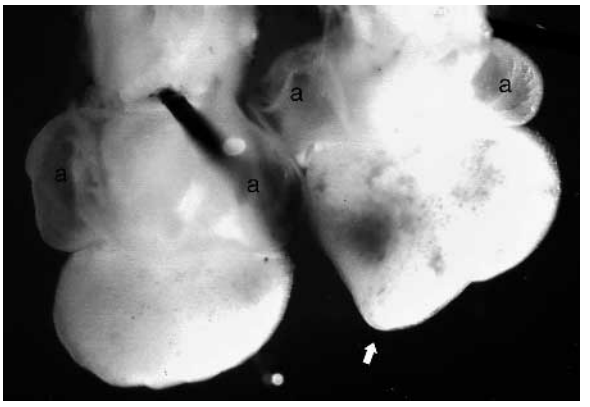

Fig. 6. Exogenous Gax expression results in morphological abnormalities. The dorsal surfaces of virus-treated hearts as seen by stereomicroscopy after 96 hours of incubation (stage 28/29). The AdRSVGax-infected heart (left) was smaller and lacked the sharp ventricular apex (arrow) present in the control (AdRSVGal-infected) heart (right). The hearts are approximately 2×2 mm. a, atrial chambers.

normal heart development (Donehower et al., 1992). The forced expression of p53 via this adenoviral delivery system did not affect the percentage of myocytes that were PCNA positive at 48 hours or 72 hours after virus application (52±7%, n=2 and 57±5%, n=3, respectively, P>0.05), demonstrating the specificity of Gax in inhibiting myocyte proliferation.

Forced precocious Gax expression causes heart abnormalities

To determine the effect that the precocious expression of Gax protein in the tubular heart, and subsequent inhibition of myocyte proliferation, has on cardiac morphogenesis, the morphologies of AdRSVGax-infected hearts were compared to hearts in which a control protein (β-gal) or no protein were expressed. A large proportion of the AdRSVGax-infected hearts exhibited morphologic abnormalities evident within 3 to 4 days (stages 27-31) of adenovirus application, while the control hearts appeared normal (Fig. 6). Most obvious was the failure of the ventricle to develop a pointed apex evident by steromicroscopic analysis (×10-×63 magnification) in 7 of 9 AdRSVGax-infected hearts. Scanning electron microscopic (SEM) observation of these intact hearts confirmed the stereomicroscope analysis (Fig. 7). Evident was ventricular hypoplasia marked by smaller ventricles and rounded ventricular apices. In addition, SEM observation revealed that AdRSVGax-infected hearts lacked the rounded swelling of the right ventricular, bulbis cordis region. The atria of two more severely affected hearts appeared smaller than those of controls, while in the more mildly affected hearts, both left and right atria were similar in size to the controls. The outflow tract in the mildly affected hearts achieved the normal central position between the two equal-sized atrial chambers.

The internal structures of a subset of the AdRSVGax-infected and control hearts were observed by SEM after slicing open the ventricular chambers (Fig. 7). The trabeculae of the AdRSVGax-infected hearts appeared shorter and thicker or coarser than controls, with a thinner musculature within the wall or compact zone of the left ventricle. The maturation of the interventricular septum (IVS), which involves coalescence

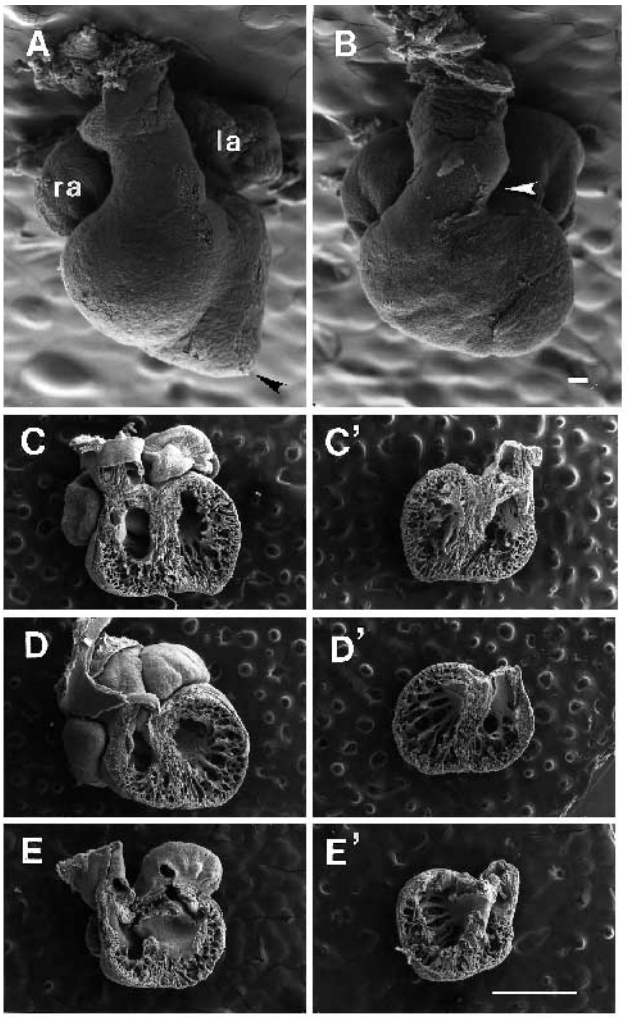

Fig. 7. Scanning electron microscopy of AdRSVGax-exposed hearts. Frontal view of an (A) AdRSVGal stage 28 control heart and (B) AdRSVGax stage 29 heart. AdRSVGax hearts lacked the pointed ventricular apex (black arrowhead) and the bulging right ventricle present in a younger control heart. Also noted in the AdRSVGax heart was a constriction in the outflow tract (white arrowhead). In hearts sliced in a frontal plane (C-E), the inner structures of control hearts (C,C′) were compared to those of AdRSVGax hearts (D,D′,E,E′) The front (C,D,E) and back halves (C′,D′,E′) from the same heart are shown. AdRSVGax-treated hearts had thinner ventricular walls, shorter and thicker trabeculae, and less dense and less organized IVS tissue, especially towards the apex. ra, right atrium; la, left atrium. Bar, (A,B) 0.1 mm; (C-E′) 1 mm

of trabecular leaves, appeared delayed with the apical region of the IVS splayed rather than compact.

DISCUSSION

Homeodomain proteins were first identified as regulators of the body plan, as evidenced by the homeotic transformations that occurred in *Drosophila* when these genes were mutated (Krumlauf, 1994; Lawrence and Morata, 1994). The homeodomain genes are also good candidate genes for determining the form of vertebrate organs. In this study, we examine the

expression of the Gax homeodomain protein during heart development and the effects of forced precocious expression of this protein in the heart. The results suggest that Gax plays a role in defining the size and shape of the heart by limiting embryonic myocyte proliferation.

Pattern of expression of endogenous *Gax* and other transcription factors

Gax was originally cloned from an adult rat aorta cDNA library (Gorski et al., 1993), and subsequently shown to be homologous to murine and *Xenopus* Mox-2 (Candia et al., 1993). Its pattern of expression during the life of the animal is complex, and is restricted both spatially and temporally. Expression of mRNA transcripts in early *Xenopus* embryos is restricted to the mesoderm (Candia and Wright, 1995) while, in the mouse, the mRNA and protein are detected in mesodermally derived cardiac, skeletal and smooth muscle tissues as well as in neuroectodermal derivatives (Skopicki et al., 1997). Here we focused our analysis on Gax protein expression and function in heart development. The protein was detected by immunohistochemistry in the nuclei of a subset of myocardial cells relatively late in development, i.e. stage 33 and later. Confocal microscopy is consistent with these nuclei belonging to cardiomyocytes, though other cells, most likely vascular, were also positive. This pattern of expression parallels that seen in the mouse heart, in which Gax protein was evident at day 15.5 p.c. in the nuclei of a subset of myocardial cells, and was not detected in heart homogenates by western blotting until birth (Soonpaa et al., 1996). Thus, in both mammals and birds, the Gax homeodomain protein is detected in the heart late in development, after many of the structures of the heart have formed.

Gax protein appears to be somewhat unique as a homeobox protein and putative transcription factor that appears late in heart development. This contrasts with other tissue-specific transcription factors, such as the homeodomain protein Nkx (Csx) family (Lee et al., 1996; Schultheiss et al., 1995; Evans et al., 1995; Tonissen et al., 1994; Lints et al., 1993; Bodmer, 1993), the MADS family MEF-2 (Edmondson et al., 1994), bHLH family dHAND and eHAND (Srivastava et al., 1995), and TEF-1 (Chen et al., 1994), which are present early and critical in the initial stages of heart morphogenesis (Rossant, 1996). Interestingly, Gax mRNA transcripts were detected by in situ hybridization at the earliest stages of heart morphogenesis, yet the protein is not detected in the nucleus as a putative transcription factor until late in heart development (Skopicki et al., 1997; and this study). These results suggest the presence of potent post-transcriptional regulation, as is also observed in the MEF-2 family of genes (Suzuki et al., 1995), and the nature of which remains to be identified.

Proliferation/development of cardiomyocytes

The size that the heart attains is influenced largely by the extent of myocyte proliferation prior to birth and myocyte hypertrophy after birth. These processes are strictly regulated, so that the number of myocytes and size of the mature heart is normally uniform within species. The tubular heart undergoes tremendous growth due to myocyte proliferation, which is dependent upon the N-myc oncogenic transcription factor. In transgenic mice in which this protein is inactivated, the hearts are hypoplastic and the ventricular myocardium thinned (Moens et al., 1993). These mice die at 12.5 days p.c., which is 3 days prior to the appearance of Gax in the myocardium (Skopicki et al., 1997).

This late appearance of Gax, at a time when myocyte proliferation declines (Soonpaa et al., 1996; Mikawa et al., 1992; Jeter and Cameron, 1971) and long after the myogenic gene program has been activated (Chien et al., 1993), suggested that it might participate in cardiomyocyte withdrawal from the cell cycle. Evidence to support this hypothesis was obtained by the forced expression of Gax earlier in development, in cardiomyocytes that are highly proliferative. It was demonstrated that clones of ventricular myocytes of the AdRSVGax-infected hearts reached a smaller size. This assay used co-transfection of the test (Gax expression) virus and a clonal marker (β-gal virus), an approach that was feasible due to the high transfection efficiency of adenovirus (Fisher and Watanabe, 1996). In another study, a recombinant retrovirus was used in which a single construct expressed both the reporter (β-gal) protein and a dominant negative FGF receptor (Mima et al., 1995). Reduced clone size was demonstrated, indicating the importance of that growth factor signaling pathway in the proliferation of embryonic cardiomyocytes in the first week of chicken heart morphogenesis. Several studies have demonstrated epicardial to endocardial gradients within the heart of myocyte proliferation (Mikawa et al., 1992; Jeter and Cameron, 1971) and in the distribution of growth factors such as FGF (Consigli and Joseph-Silverstein, 1991), as well as some regional variation in myocyte proliferation (Thompson et al., 1990). The analyses performed in the current study, and in the study mentioned above, were not able to determine the relationship between the epicardial-to-endocardial proliferative gradient and Gax or FGF expression, respectively. It is intriguing to note, however, that in vitro the expression of Gax is extinguished by growth factor stimuli that induce cell proliferation (Gorski et al., 1993). In the current study, the reduced cell number resulting from the forced expression of Gax was shown to be due to myocyte withdrawal from the cell cycle as evidenced by a reduction in the percentage of cells that were positive for a biochemical marker of proliferation, PCNA. This effect was observed when Gax was expressed under the control of a viral promoter; whether there is a threshold level required for this effect is not known. The mechanism by which Gax exerts this anti-proliferative effect in the developing heart has yet to be determined.

These results suggest a role for the Gax homeodomain protein relatively late in heart morphogenesis in bringing cadiomyocyte proliferation to an end and limiting the size of the heart. This contrasts with the apparent role of the Nkx family of homeodomain proteins. In experiments in which Nkx 2.3 and/or Nkx 2.5 cRNA were injected into *Xenopus* cells, hyperplastic, enlarged hearts were observed (Chen and Fishman, 1996; Cleaver et al., 1996). These data were interpreted as showing that Nkx functions to maintain cells in the cardiac field in the myocytic lineage, though no direct measurements of cell proliferation were made to test the alternative explanation that Nkx stimulates proliferation.

While a great deal remains to be learned about the regulatory network of cardiomyocyte proliferation, cell cycle arrest and differentiation, it is clear that the situation is different from skeletal muscle, in which a family of bHLH myogenic determinant proteins cause proliferative myoblasts to differentiate and to exit the cell cycle (reviewed in Olson et al., 1995;

Tapscott et al., 1988)). Heart muscle cells throughout early development are both differentiated and proliferating, making it unlikely that a single factor or class of factors is responsible both for differentiation and cell-cycle withdrawal. In this regard, we have previously shown that the transcription of Gax in the developing heart is in part controlled by MEF-2 (Andres et al., 1995), a second class of myogenic determinant genes that are expressed at the earliest stages of heart differentiation (Edmondson et al., 1994) and activate the muscle gene program (Yu et al., 1992). These data support a hypothetical model in which activation of the myogenic gene program leads to cardiomyocyte cell cycle arrest via the Gax homeodomain protein.

Forced expression of Gax perturbs morphogenesis

The morphologic consequence of forced Gax expression, ventricular hypoplasia, is consistent with the observed inhibition of proliferation. AdRSVGax-treated hearts differed from controls not only in size but also in the formation of ventricular structures. The shapes of the ventricles were rounder and missing sharp apices, the ventricular walls were thinner, and maturation of the trabeculae and interventricular septae were delayed. The effect of Gax on ventricular morphogenesis may be due to the timing of the expression of the exogenous protein, which coincides with the period of rapid ventricular growth and morphogenesis. This period includes stages 25 to 28 when a marked change occurs in the external morphology of the chicken ventricle (De La Cruz et al., 1972). The rounded ventricle becomes pointed at the apex and lengthens and the future right ventricle or bulbis cordis swells. This is also a time when trabeculae grow by addition of cells from the highly proliferative ventricular wall into the base of the trabeculae (Harh and Paul, 1975). The interventricular septum matures during this period, in a process in which the trabecular leaves coalesce (Ben-Shachar et al., 1985). Therefore, we propose a scenario in which Gax misexpression in the ventricular wall reduces proliferation in these normally highly proliferative cells. This reduces the total number of myocardial cells in the ventricle wall that would have contributed to the trabeculae, stunts the growth of the individual trabeculae and, thereby, affects the formation of the interventricular septum.

In summary, a molecular mechanism by which the heart terminates a rapid period of hyperplastic growth appears to involve the nuclear presence of the Gax homeodomain protein in myocytes at a late stage in morphogenesis. The forced precocious nuclear expression of Gax protein causes cardiomyocytes to prematurely exit the cell cycle, resulting in perturbations in morphogenesis that reflect the reduction in myocyte proliferation. The mechanisms that insure that this process occurs at the appropriate time during normal heart development remain to be determined.

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