Cell history determines the maintenance of transcriptional differences between left and right ventricular cardiomyocytes in the developing mouse heart

Robert G. Kelly*,‡, Marguerite Lemonnier, Stephane Zaffran, Andrew Munk and Margaret E. Buckingham‡

CNRS URA 2578, Department of Developmental Biology, Pasteur Institute, 25 Rue du Dr Roux, Paris 75015, France

*Present address: Department of Genetics & Development, Columbia University, 701 West 168th Street, New York, NY 10032, USA

‡Authors for correspondence (e-mail: margab@pasteur.fr; rkelly@pasteur.fr)

Accepted 6 August 2003

Journal of Cell Science 116, 5005-5013 © 2003 The Company of Biologists Ltd

doi:10.1242/jcs.00824

Summary

The molecular mechanisms that establish and maintain transcriptional differences between cardiomyocytes in the left and right ventricular chambers are unkown. We have previously analysed a myosin light chain 3f transgene containing an *nlacZ* reporter gene, which is transcribed in left but not right ventricular cardiomyocytes. In this report we examine the mechanisms involved in maintaining regionalised transgene expression. Primary cardiomyocytes prepared from left and right ventricular walls of transgenic mice were found to maintain transgene expression status in culture. However, similar cultures prepared from nontransgenic mice or rats show uniform expression after transient transfection of *Mlc3f* constructs, suggesting that the mechanism responsible for differential expression of the transgene between left and right ventricular cells does not operate on transiently introduced molecules. These data suggest that developmental cell history determines transgene expression status. Maintenance of transgene expression status is regulated by a cell-autonomous mechanism that is independent of DNA methylation, trichostatin A-sensitive histone deacetylation and miss-expression of transcription factors that are expressed in the left or right ventricles of the embryonic heart. Parallels between *Mlc3f* transgene repression in right ventricular cardiomyocytes and polycomb-mediated silencing in *Drosophila* suggest that *Mlc3f* regulatory sequences included on the transgene may contain a cellular memory module that is switched into an on or off state during early cardiogenesis. Epigenetic mechanisms may therefore be involved in maintaining patterning of the mammalian myocardium.

Key words: Cardiomyocytes, Transcriptional repression, Transgenic mice

Introduction

The establishment and maintenance of transcriptional diversity during organogenesis is a key feature of embryonic development. Within the developing heart, myocardial cells in different cardiac chambers exhibit differences in gene expression, which reflect functional compartmentalisation (Christoffels et al., 2000). These transcriptional differences are established during early heart development and prefigure the formation of the specialised left and right atrial and ventricular chambers, which direct separate systemic and pulmonary blood flows.

Molecular analysis of the regulatory circuits controlling cardiomyocyte diversity has identified a small number of *cis*-acting motifs and *trans*-acting factors involved in atrial versus ventricular identity and in differential gene expression between cells of the ventricular and atrioventricular canal myocardium (Wang et al., 2001; Habets et al., 2002). In addition, several *cis*-acting elements active in cardiomyocytes of either the left or right ventricle have been defined in transgenic mice (Schwartz and Olson, 1999; Kelly et al., 1999). Although the *trans*-acting factors that regulate such transgenes remain unknown, mutational analysis of transcription factors expressed throughout the heart has in some cases revealed compartment-restricted roles during early development (Lyons et al., 1995; Lin et al., 1997). A small number of cardiac transcription factors, in particular the basic helix-loop-helix (bHLH) proteins Hand1 and Hand2, and the T-box-containing regulatory factor Tbx5, show left/right differences in expression pattern in the embryonic ventricles, and the generation of null alleles in the *Hand1*, *Hand2* and *Tbx5* genes has shown that these factors are important in chamber morphogenesis (Srivastava et al., 1997; Firulli et al., 1998; Riley et al., 1998; Bruneau et al., 2001). Despite these studies, the molecular mechanisms that initiate and maintain left versus right ventricular specific gene expression are poorly understood. In particular, little is known about the factors regulating transcriptional differences between left and right ventricular chambers at later developmental stages. How left/right ventricular transcriptional differences are maintained is of major interest, as the fundamentally different roles of the two ventricles become apparent only on the separation of pulmonary and systemic circulatory systems at birth. Furthermore, in the adult, cardiac hypertrophy elicits changes in gene expression that differ in the left versus right ventricular free walls (Vikstrom et al., 1998).

Certain transgenes have provided valuable indicators of transcriptional differences between cells of the myocardium. Regionalised expression of an *Mlc3f* promoter *nlacZ* transgene is established between embryonic days (E) 8 and 10 (Franco et al., 1997). At E10.5 this transgene is expressed in the right atrium and left ventricle, in a similar manner to the endogenous *Mlc3f* gene (Kelly et al., 1998). Subsequently, the *Mlc3f-nlacZ-2* transgene provides a stable regional marker for cardiomyocytes in the left ventricle from the completion of cardiac looping through to the adult (Kelly et al., 1999). Together with other transgenic studies, analysis of *Mlc3f* transgene expression suggests that transcriptional regulation in the mouse heart is modular such that distinct *cis*-acting elements regulate spatial and temporal expression patterns in the developing myocardium (Firulli and Olson, 1997; Kelly et al., 1999; MacNeill et al., 2000).

The *Mlc3f-nlacZ-2* transgene provides a tool with which to investigate the mechanisms that initiate transcriptional differences between cardiomyocytes and those that maintain such differences throughout subsequent development. We initiated a series of experiments to investigate the mechanisms involved in the maintenance of *Mlc3f* transgene regionalisation using primary ventricular cardiomyocyte cultures. We show that left and right ventricular cardiomyocytes maintain their positional identity ex vivo as revealed by *Mlc3f* transgene expression. However, the same *Mlc3f* promoter constructs are expressed equally in left and right cardiomyocytes after transient transfection into nontransgenic cultures, suggesting that the maintenance of regional transgene expression is mediated by different mechanisms from those that establish regionalisation. We show that the mechanism mediating repression of *Mlc3f* transgene activity in right ventricular cardiomyocytes is cell autonomous and independent of both global DNA methylation and trichostatin A-dependent histone deacteylation. Furthermore, *Mlc3f* transgene expression in such cultures is unaffected by the overexpression of exogenous transcription factors that show left/right regionalisation in the embryonic heart. These observations suggest that developmental cell history plays an important role in the maintenance of *Mlc3f* transgene expression status.

Materials and Methods

Rat and mouse primary cardiocyte cultures

Cardiac myocytes were obtained from fetal Wistar rats (Janvier, France) and from *Mlc3f-nlacZ* transgenic and nontransgenic mouse embryos. *Mlc3f-nlacZ-2* and *Mlc3f-nlacZ-2E* transgenic mice have been described previously (Kelly et al., 1995; Kelly et al., 1998). Homozygous males were crossed with nontransgenic females to obtain 100% transgenic litters. Female rats or mice were killed at midday on day 18 of gestation using carbon dioxide inhalation and cervical dislocation, according to approved protocols. Mouse ventricular cardiocytes were prepared from dissected free walls of right or left ventricles according to a previous protocol (Knowlton et al., 1995), with slight modifications. Cells were dissociated under gentle shaking in 1× trypsin (T4674 Sigma) in Ads (116.4 mM NaCl, 5.4 mM KCl, 5.4 mM glucose, 10.5 mM NaH_2PO_4, 405.7 μM $MgSO_4$, 20 mM HEPES pH 7.3). Trypsin was inhibited by adding decomplemented newborn calf serum (Gibco-BRL). Cells were recovered by centrifugation and kept at 37°C in newborn calf serum. Fresh enzyme was added to the tissue until completely dissociated. Cells were then pooled and washed twice in newborn calf serum. Cells were plated in 6 cm dishes at a density of $7\text{-}8\times10^5$ per plate in plating medium containing newborn calf serum and grown at 37°C in 5%CO_2. Medium was replaced after 18 hours, at which stage beating cardiomyocytes were observed.

In co-culture assays, 10^4 cells dissociated from free right or left ventricular walls of transgenic embryos were plated onto gelatinised spots in 3 cm plates together with various amounts (10^2 to 10^4) of cells prepared from either right or left free walls of nontransgenic embryos. Following fixation and staining with X-gal, the number of blue nuclei was determined in several fields and expressed as mean ± s.e.m. (n=5). Gelatinised spot cultures were also used for the experiments involving adenoviral infection.

β-galactosidase staining and immunocytochemistry

Cells were washed twice with PBS and fixed in freshly prepared 4% paraformaldehyde for 5 minutes at room temperature. β-galactosidase staining was performed on fixed cells by incubation in X-gal solution for different lengths of time at 37°C as previously described (Sanes et al., 1986). For transgenic cultures the average number of β-galactosidase-positive nuclei per field was scored for at least five fields. Student's *t*-test was used to test significant differences between means.

Immunostaining for β-galactosidase, α-actinin and acetylated Histone H4 were performed as described (Cossu et al., 1996). Following fixation, permeabilisation and blocking, the cells were incubated with a monoclonal anti-mouse and a polyclonal anti-rabbit antibody for 2 hours (see below for dilutions), followed by incubation with a Texas red-coupled anti-mouse IgG antibody (1/200) (Jackson Labs) and a fluorescein-coupled anti-rabbit IgG antibody (1/200) (Jackson Labs). Before mounting in Immuno Floure mounting Medium (ICN), nuclei were stained with Hoechst 33258. Monoclonal anti-α-actinin (dilution 1/200) was obtained from Sigma, monoclonal anti-β-galactosidase (dilution 1/100) from Molecular Probes and polyclonal anti-acetylated histone H4 (dilution 1/200) from Upstate Biotechnology.

Transfections, luciferase and β-galactosidase assays

Cardiomyocytes were transfected by the calcium phosphate technique as described by Biben et al. (Biben et al., 1994). All *Mlc3f* plasmids used have been described previously (Kelly et al., 1995; Kelly et al., 1997). Ten micrograms of *lacZ* reporter construct was used, together with 0.5 μg of a reporter gene containing the Rous Sarcoma virus long terminal repeat linked to the luciferase gene (*RSVluc*) for normalisation of transfection efficiency. When required, total input DNA was maintained at a constant level by adding the appropriate amount of BlueScript vector (Stratagene). Sixty hours after transfection, cardiocytes were washed twice with PBS, collected in 40 mM pH 7.5 Tris, 150 mM NaCl and 1 mM EDTA, and lysed by three freeze-thaw cycles as reported elsewhere (Biben et al., 1994). β-galactosidase activity was quantified using a GalactoLight (Tropix) chemiluminescent kit and luciferase activity was measured as described elsewhere (Kelly et al., 1997) for 10 seconds in a Berthold luminometer. Each transfection was performed at least three times with different plasmid DNA preparations.

Drug treatment

Primary cardiomyocyte cultures were plated, and after overnight culture in standard conditions the medium was replaced with fresh medium (control) or with medium containing 2 mM sodium butyrate (Sigma) and 100 μM trichostatin A (Sigma), 4 μM angiotensin (Sigma) or 10 μM phenylephrine (Sigma). Culture medium and test compounds were replaced every day. Cultures were analysed 4 days after the beginning of treatment.

Analysis of genomic methylation status

DNA was prepared from different tissues of 2-month-old transgenic

mice and analysed by Southern blot hybridisation. Ten micrograms of DNA were digested with restriction endonucleases *Hpa*II or *Msp*I and subjected to electrophoresis. Transfer onto a Hybond N+ membrane (Amersham) and hybridisation were as reported previously (Kelly et al., 1995), using a 2 kb *Cla*I-*Bam*HI *lacZ* hybridisation probe.

Adenoviral infection

Adenovirus was generated as previously described by Hedlund et al. (Hedlund et al., 1998). Briefly, full-length murine *Hand* and *Tbx5* cDNAs were obtained by polymerase chain reaction (PCR) amplification using a high-fidelity Taq polymerase (Clontech) and cloned in a pGEMT vector (Promega). All numbers on *Hand1*, *Hand2* and *Tbx5* cited in this section refer to the GenBank sequence NM_008213, AF034435 and XM_132278, respectively. For the *Hand1* fragment (+362/+1012) primers *Hand1Forw* ATAAGAATGCGGCCGCATG-AACCTCGTGGGCAGCTACGCAC and *Hand1Rev* GCTCTAGA-TCACTGGTTTAGCTCCAGCGCCCAG were used. For the *Hand2* fragment (+8/+1672) primers *Hand2Forw* ATAAGAATGCGGCCG-CATGAGTCTGGTGGGGGGCTTTCCCCAC and *Hand2Rev* GCT-CTAGATCACTGCTTGAGCTCCAGGGCC were used. For the *Tbx5* fragment (+193/+1749) primers *Tbx5Forw* GGAATTCGCGGCCG-CATGGCCGATACAGATGAGGGCTTTGG and *Tbx5Rev* GGTCA-GCCTCTAGATATTCTCACTCCACTCTGGC were used. All PCR products were sequenced to confirm their predicted composition. Subsequently, cDNAs were cloned in pAdTrack-CMV (cytomegalovirus) vector and co-transfected with the pAdEasy adenoviral plasmid into HEK 293 cells to generate high-titre viral stocks. After repeated amplification in HEK 293 cells, viral titres were high enough (10^9 plaque-forming units) to use for gene transfer into primary cardiomyocyte cultures. The recombinant adenoviruses contain two independent CMV-driven transcription units, one driving the cDNA of interest and one driving a green fluorescent protein (GFP) reporter gene. One to ten microlitres of concentrated viral stock were used for adenoviral infection in 100 μl medium.

Expression vector activity was tested in HEK 293 cells using an atrial natriuretic factor (*ANF*)-luciferase reporter construct, as the *ANF* promoter is known to be a target of Tbx5 (Habets et al., 2002). The efficiency of transfection was normalised with a *CMV-nlacZ* construct. The adeno-GFP vector and *VP16-Tbx2* were used as negative and positive controls, respectively. In three independent transfections, each carried out in duplicate, the negative control gave an average figure of fivefold over background, compared with 60-fold for the positive control and 20-fold for the *Tbx5* expression vector, thus showing that the latter is active.

Results

Regional reporter gene expression is maintained in primary ventricular myocytes from transgenic mice

We have previously shown that elements within 2 kb upstream of the transcriptional initiation site of the *Mlc3f* gene direct reporter gene expression to the right atrium and left ventricle (Kelly et al., 1998) (Fig. 1A). To investigate the mechanism by which regional *Mlc3f* transgene expression is maintained, reporter gene activity was analysed in primary ventricular cardiomyocyte cultures prepared from free right and left ventricular walls of late fetal (E17.5-18.5) *Mlc3f-nlacZ-2* hearts. Beating cardiomyocytes, which were positive for the sarcomeric protein α-actinin, formed the predominant cell type in these cultures after 20 hours (Fig. 1B), although nonmyocardial cell types were also present (arrowheads in Fig. 1B).

Mlc3f-nlacZ-2 transgene expression was monitored by X-gal incubation after 2 days, 1 week and 3 weeks of culture.

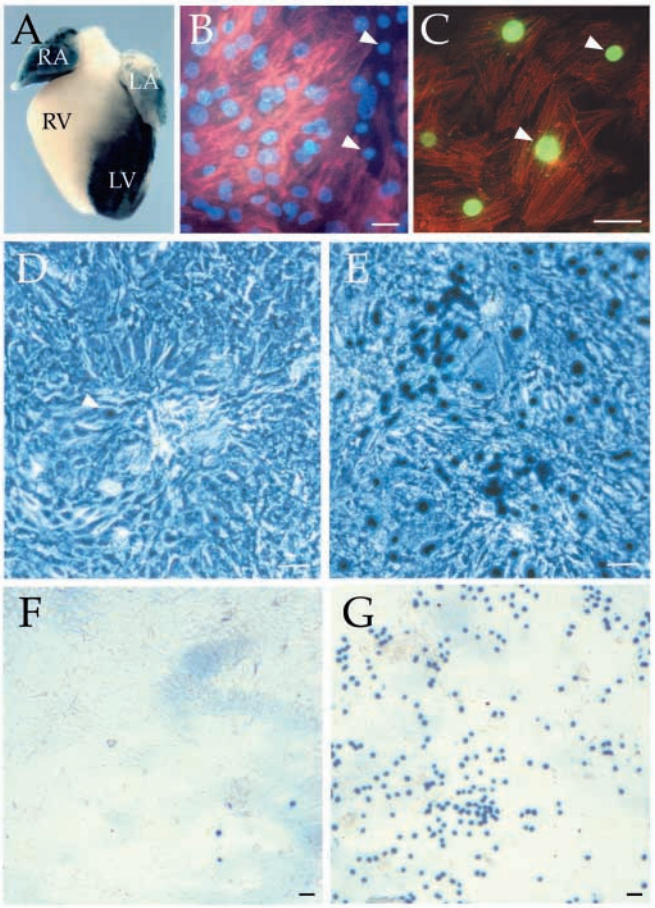

Fig. 1. *Mlc3f-nlacZ* transgene expression status is maintained ex vivo. (A) Expression pattern of the *Mlc3f-nlacZ-2* transgene in a neonatal heart, showing β-galactosidase activity restricted to the right atrial and left ventricular compartments. LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle. (B) Primary ventricular culture following immunocytochemistry to detect α-actinin (red) and Hoechst staining to detect nuclei (blue). Most cells in the cultures are α-actinin positive cardiomyocytes; a minority of nonmyogenic cells are also present (arrowheads). (C) Co-immunohistochemistry of primary left ventricular cardiomyocytes showing that β-galactosidase activity (green) is specific to cardiomyocyte nuclei (arrowhead), identified by α-actinin expression (red). (D) Phase contrast image of X-gal-stained primary ventricular cultures showing that few β-galactosidase-positive cardiomyocytes (arrowhead) are observed in right ventricular free-wall cultures, whereas (E) many β-galactosidase-positive cardiomyocytes are observed in left ventricular free-wall cultures. (F, G) Low magnification bright-field view of right (F) and left (G) primary ventricular cultures after X-gal staining. Scale bars: 10 μm (B-C), 25 μm (D-G).

Co-immunocytochemistry with anti-α-actinin and anti-β-galactosidase antibodies confirmed that transgene expression was specific to differentiated cardiomyocytes (Fig. 1C). At all timepoints analysed many more β-galactosidase-positive nuclei were observed in left than right ventricular cardiomyocyte cultures, consistent with the expression pattern of the *Mlc3f-nlacZ-2* transgene in vivo (Fig. 1D-G). The numbers of β-galactosidase-positive nuclei observed per field were at least tenfold higher in left compared with right ventricular cultures ($P<0.001$; Table 1). Consistent with this

Table 1. Transgene expression in ventricular free-wall myocyte cultures treated with trichostatin A or with hypertrophic agonists angiotensin II and phenylephrine

	No. β-gal +ve nuclei*	s.e.m.	β-gal activity†	s.e.m.
Trichostatin A				
Right ventricle	2.7	0.6	1.2	0.3
Left ventricle	39.4	3.0	13.9	3.1
Right ventricle+TSA	3.2	1.1	0.7	0.1
Left ventricle+TSA	29.6‡	2.6	14.0	2.7
Angiotensin II and phenylephrine				
Right ventricle	1.8	0.5		
Left ventricle	37.8	2.9		
Right ventricle+AT	1.6	0.4		
Left ventricle+AT	19.5‡	3.2		
Right ventricle+Ph	1.8	0.5		
Left ventricle+Ph	30.5	4.8		

*β-gal +ve nuclei per 20× field (average of two experiments each of ten or more fields) with standard error of the mean (s.e.m.).

†β-gal activity [relative light unit (RLU)/10^4/0.7 μl extract/protein concentration (μg/ml)].

‡Decrease in β-gal +ve nuclei due to overall reduction in cardiomyocyte number.

Abbreviations: AT, angiotensin II; β-gal, β-galactosidase; Ph, phenylephrine; TSA, trichostatin A.

observation, quantification of β-galactosidase activity in left and right cultures using a chemiluminescent assay revealed activities at least 12-fold higher in left than in right cultures (P<0.01; Table 1), comparable to >18-fold levels of β-galactosidase activity per μg protein in extracts of dissected left versus right ventricular walls (Kelly et al., 1998). These results show that cardiomyocyte transgene expression status is maintained in primary culture and is independent of functional or morphological constraints imposed by the in vivo environment. Similar results were obtained for a second transgenic line showing a right atrial/left ventricular expression pattern (*Mlc3f-nlacZ-2E* line 1), confirming dependence on regulatory sequences within the *Mlc3f* promoter rather than sequences adjacent to the transgene integration site (data not shown).

Mlc3f constructs are expressed equally in transient transfection assays with left and right nontransgenic ventricular myocytes

The above observations suggest that myocytes retain their regional identity in culture and that transient transfection of nontransgenic primary cultures might be a rapid way to define *cis*-acting sequences that are active in left but not in right ventricular myocytes. Primary cardiomyocyte cultures were prepared from free left and right ventricular walls of nontransgenic E17.5-18.5 mouse or E18.5 rat hearts. The day after plating the cardiomyocytes were transfected with *Mlc3f-nlacZ-2* plasmid and reporter gene activity was assayed 4 days after transfection. In contrast to the results obtained with left and right cultures from transgenic hearts, equal numbers of β-galactosidase-positive myocytes were observed after transient transfection of either left or right ventricular cultures prepared from nontransgenic mice or rats (Fig. 2A-D). Quantitative analysis of reporter gene activity revealed a ratio of 1 for normalised β-galactosidase activity in left over right

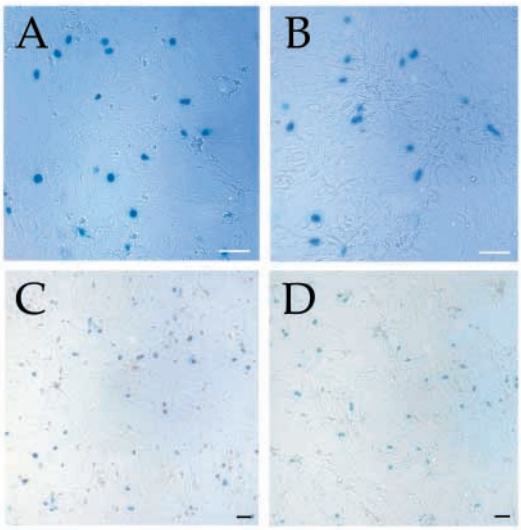

Fig. 2. Nontransgenic left and right primary ventricular cultures after transient transfection. (A,B) Phase contrast image of X-gal-stained rat primary cultures prepared from right (A) and left (B) free ventricular walls after transfection with the *Mlc3f-nlacZ-2* construct. Similar numbers of β-galactosidase positive nuclei are observed in left and right cultures. (C,D) Low magnification bright-field view of right (C) and left (D) rat primary cultures after transfection with the *Mlc3f-nlacZ-2* construct and X-gal staining. Scale bars: 25 μm.

ventricular cultures (Fig. 3A). Similarly, a ratio of 1 was also observed for other *Mlc3f* constructs including either a downstream skeletal muscle enhancer element (*Mlc3f-nlacZ-2E)*, or 7 kb of the first *Mlc1f/3f* intron (*Mlc3f-nlacZ-9*), as well as an RSV-*lacZ* construct (Fig. 3A). Thus, despite the observation that the *Mlc3f-nlacZ-2* reporter gene construct is always expressed in a left ventricular dominant profile in transgenic mice, and that cardiomyocytes from *Mlc3f-nlacZ-2* transgenic mice maintain their positional identity ex vivo, transiently transfected *Mlc3f* constructs are active in cultures of both left- and right-derived cardiomyocytes. These results suggest that transcription of the *Mlc3f-nlacZ-2* transgene is repressed in right ventricular primary cultures through a mechanism that does not act on constructs introduced by transient transfection.

Transiently transfected promoter constructs differ in several ways from stably integrated transgenes. On transient transfection template copy number is higher than that of most transgenes, the template is not integrated into a mouse chromosome and the template has not been present throughout the preceding steps of development. We tested the hypothesis that the presence of high template copy numbers saturates the activity of a repressor of *Mlc3f* promoter activity in right ventricular cardiomyocytes. Decreasing amounts of *Mlc3f-nlacZ-2* DNA were transfected into primary cardiomyocytes, in the presence of vector DNA to maintain total transfected DNA at 10 μg. No significant differences in expression levels in right versus left cultures were observed even when 100-fold less *Mlc3f-nlacZ-2* DNA (100 ng) was used and β-galactosidase activity approached background levels (Fig. 3B). Because no transcriptional differences were apparent even at low template concentrations, these results suggest that transcription from the *Mlc3f* promoter after transient transfection into right ventricular

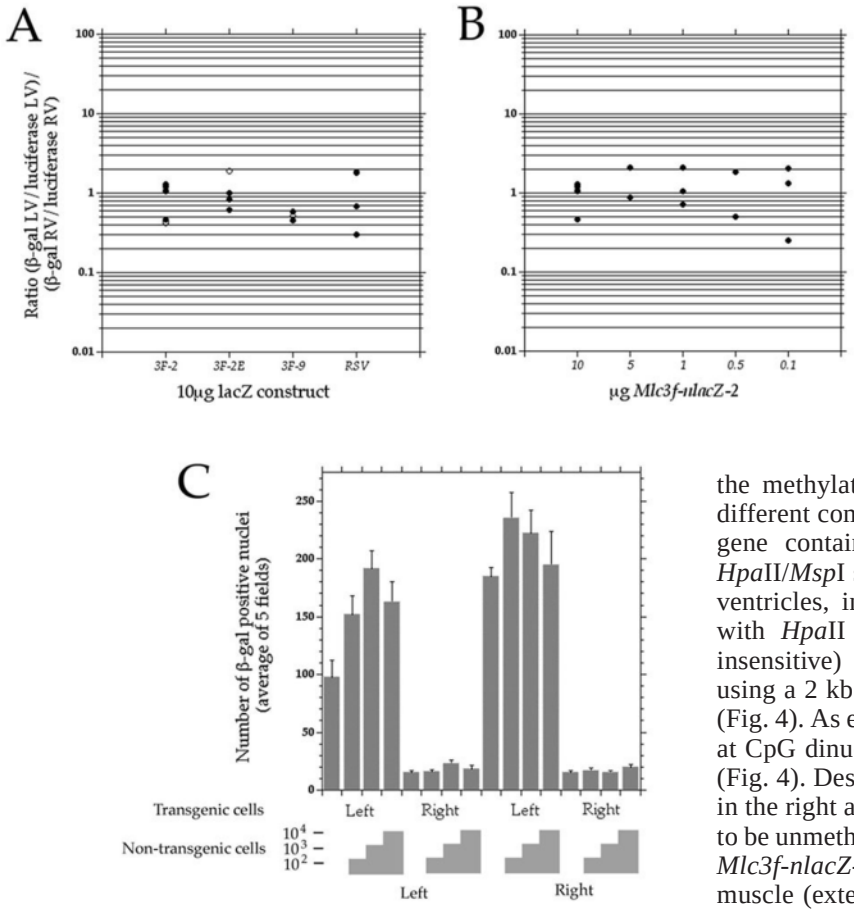

Fig. 3. β-galactosidase activity in primary ventricular myocyte cultures. (A) The ratio of β-galactosidase activity in left over right cultures is approximately 1 for different *Mlc3f* constructs and RSV-*lacZ* reporter genes after transfection into either rat (black circles) or nontransgenic mouse (open circles) primary ventricular free-wall cultures. (B) Decreasing the amount of *Mlc3f-nlacZ-2* reporter gene does not change the ratio of β-galactosidase activity in left or right nontransgenic cultures after transient transfection. (C) β-galactosidase activity in transgenic cultures is cell autonomous. Co-culture with increasing amounts of rat cardiomyocytes from the opposite ventricle does not change the number of β-galactosidase-positive cells in either left or right ventricular cultures. The average number of β-galactosidase-positive nuclei over five fields plus s.e.m. is shown for each co-culture.

cardiomyocytes is not due to copy-number-dependent suppression of the activity of a right ventricular repressor.

We subsequently asked whether transgene expression status in right and left ventricular cultures was maintained by cell-autonomous mechanisms or whether it was the result of cell-cell signalling events. To distinguish between these possibilities, co-culture experiments were carried out using left and right free ventricular wall cultures from transgenic mice in the presence of increasing amounts of nontransgenic left or right ventricular cardiomyocytes. No significant decrease in the number of β-galactosidase-positive nuclei in left ventricular cultures was observed in the presence of increasing amounts of nontransgenic right ventricular myocytes, nor was an increase in the number of β-galactosidase-positive nuclei observed in right ventricular cultures in the presence of increasing amounts of nontransgenic left ventricular myocytes (Fig. 3C).

Together, the above experiments suggest that the repressed transcriptional state of the *Mlc3f* transgene in right ventricular cardiomyocytes is maintained by a cell-autonomous mechanism that is dependent on developmental cell history.

The *Mlc3f-nlacZ* transgene is globally demethylated in all subcompartments of the heart

Transcriptional repression has been shown to be associated with hypermethylation (Newell-Price et al., 2000). We investigated the methylation status of the *Mlc3f-nlacZ-2E* transgene in different compartments of the adult heart. The *nlacZ* reporter gene contains multiple CpG dinucleotides, including 15 *Hpa*II/*Msp*I sites. Genomic DNA isolated from right and left ventricles, in addition to right and left atria, was digested with *Hpa*II (methylation sensitive) or *Msp*I (methylation insensitive) and analysed by Southern blot hybridisation using a 2 kb probe from the 3′ end of the *lacZ* reporter gene (Fig. 4). As expected, the bulk of genomic DNA is methylated at CpG dinucleotides and is therefore not digested by *Hpa*II (Fig. 4). Despite the fact that this reporter gene is active only in the right atrium and left ventricle, the transgene was found to be unmethylated in all cardiac compartments analysed. The *Mlc3f-nlacZ-2E* transgene was also unmethylated in skeletal muscle (extensor digitorum longus) where the reporter gene is active, and in other tissues where it is transcriptionally inactive, such as the kidney (data not shown). These results are consistent with a PCR-based study of the methylation status of specific CpG dinucleotides in the *Mlc3f* promoter (McGrew et al., 1996) and suggest that the mechanism of transcriptional repression in the right ventricle is independent of global DNA methylation.

The mechanism of transcriptional repression of the *Mlc3f-nlacZ-2* transgene is independent of trichostatin A-sensitive histone deacetylation and treatment with hypertrophic agonists

Transcriptional repression has also been associated with histone deacetylation (Wade, 2001). Removal of actetyl residues from histones leads to a closed chromatin configuration and transcriptional silencing. To investigate whether histone deacteylation is implicated in the transcriptional repression of the *Mlc3f-nlacZ-2* transgene in the right ventricle we treated primary ventricular cultures from transgenic mice with the histone deactylase inhibitor trichostatin A combined with sodium butyrate. Treatment was monitored by immunohistochemistry with antibodies specific to the acetylated form of histone H4. An increase in acetylation of histone H4 was observed in all nuclei on treated plates but did not significantly increase the number of β-galactosidase-positive nuclei in right ventricular cultures compared with untreated plates ($P>0.1$; Fig. 5 and Table 1). A statistically significant decrease in the number of positive nuclei in left ventricular cultures was observed after trichostatin A treatment

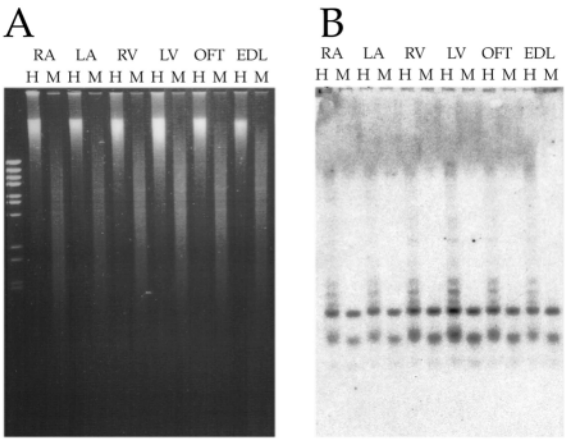

Fig. 4. *Mlc3f-nlacZ* transgenes are unmethylated in all compartments of the heart. (A) Ethidium bromide-stained gel showing restriction endonuclease digested *Mlc3f-nlacZ-2E* DNA isolated from different regions of the heart or fast skeletal muscle with *Hpa*II (H) or *Msp*I (M) endonucleases. (B) Southern blot hybridisation of the gel in A using a 2 kb *lacZ* probe, showing that the reporter gene is unmethylated to an equivalent degree in all samples. EDL, extensor digitorum longus (fast skeletal muscle); LA, left atrium; LV, left ventricle; OFT, right ventricular outlet; RA, right atrium; RV, right ventricle.

(P<0.05). Given the stability of β-galactosidase, this is likely to reflect a decrease in total cell numbers subsequent to treatment rather than extinction of transgene expression. In support of this interpretation comparison of β-galactosidase activities in extracts normalised for protein concentration revealed no statistical difference between treated and untreated cultures. These results suggest that transcriptional repression of the *Mlc3f-nlacZ-2* transgene in right ventricular cardiomyocytes is independent of trichostatin A-sensitive histone deacetylation.

We tested the possible effects of culturing left and right cardiomyocytes in the presence of hypertrophic agonists that are known to modify cardiac gene expression patterns. Angiotensin II has been reported to induce a pathway that activates the immediate early gene *c-fos* in adult cardiomyocytes, which, in turn, activates an adult-to-fetal-isoform transition of genes encoding actin, myosin and atrial natriuretic factor (Sadoshima et al., 1993). However, following treatment with angiotensin II for two days there was no significant increase in the number of β-galactosidase-positive nuclei in right ventricular cultures compared with untreated plates (Table 1). Similar results were obtained with the hypertrophic agonist phenylephrine (Table 1).

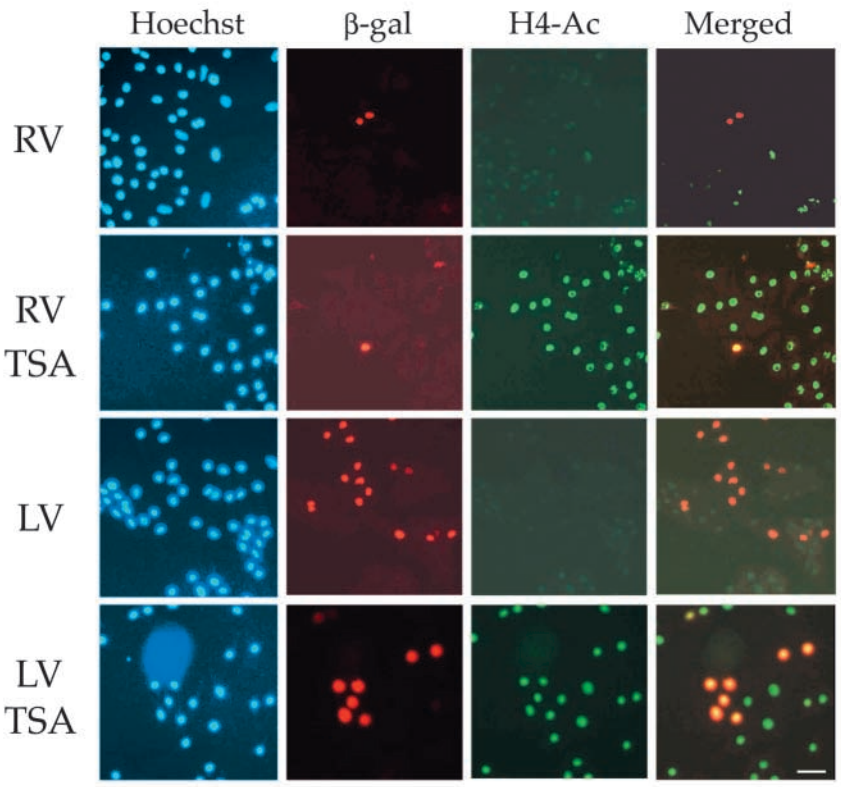

Fig. 5. Trichostatin A treatment does not modify *Mlc3f-nlacZ-2* transgene expression status. Co-immunohistochemistry of primary ventricular cardiomyocyte cultures following treatment with trichostatin A and sodium butyrate for 3 days, showing Hoechst staining to identify all nuclei per field, anti-β-galactosidase (red), anti-acetylated histone H4 (green) and the overlay of anti-β-galactosidase and anti-acetylated histone H4 (merged). Note that although the number of nuclei testing positive for acetylated histone H4 increases after trichostatin A treatment, no change in transgene expression status is observed (see also Table 1). Scale bar: 25 μm.

Mlc3f-nlacZ-2 transgene expression status in primary ventricular cultures is unaffected by overexpression of the transcription factors Hand1, Hand2 or Tbx5

Hand1 and Hand2 are bHLH-containing transcription factors that play crucial roles in ventricular development (Srivastava, 1999). These genes have a complementary expression pattern in the embryonic mouse heart, with *Hand1* being expressed in the future left ventricle and *Hand2* being expressed in the future right ventricle (Srivastava et al., 1997; Biben and Harvey, 1997). This early expression may contribute to the establishment of different transcriptional circuits in left and right ventricles; these transcription factors are downregulated in the ventricles during fetal development (Zammit et al., 2000). We asked whether miss-expressing these transcription factors in primary cultures would modify the expression status of the *Mlc3f-nlacZ-2* transgene.

Cultures from the right and left ventricular free walls of *Mlc3f-nlacZ-2* transgenic mice were transfected with expression vectors driving full-length cDNAs encoding either Hand1 or Hand2 with a myc epitope tag. Three days after transfection co-immunocytochemistry was performed with anti-myc and anti-β-galactosidase antibodies. *Hand* gene transfection did not modify transgene expression status (Fig. 6). β-galactosidase-negative, Myc-positive, right ventricular cardiomyocytes were observed after

transfection with *Hand1*, which is normally expressed in the embryonic left ventricle (Fig. 6A-C). Similarly, β-galactosidase-positive, Myc-positive, left ventricular cardiomyocytes were observed after transfection with *Hand2*, which is normally expressed in the embryonic right ventricle (data not shown). To increase transfection efficiency primary ventricular cultures were infected with recombinant adenoviruses containing *CMV-GFP* and either *CMV-Hand1* or *Hand2* cDNAs. After 4 days of culture, cells were fixed, stained with X-gal and examined by fluorescence (Fig. 6D). In all cultures the majority of cells expressed GFP, thereby indicating a high degree of adenoviral infection. However, again, transgene expression status was not modified. In cultures obtained from the left ventricular free wall, β-galactosidase-negative cells overexpressing *Hand1* and β-galactosidase-positive cells overexpressing *Hand2* were scored. The majority of cells in right ventricular cultures were β-galactosidase negative irrespective of which *Hand* gene they overexpressed (Fig. 6D). Identical results were obtained when adenoviral infection was combined with trichostatin A treatment (data not shown). Transgenic cardiomyocytes were also infected with adenoviral vectors expressing *Tbx5*, a transcription factor that is normally restricted to the embryonic left ventricle (Bruneau et al., 1999). As in the case of *Hand1* miss-expression, right ventricular cardiomyocytes infected with the *Tbx5* adenovirus did not activate the reporter gene, even in the presence of trichostatin A or after co-infection with a *Hand1* encoding adenovirus plus trichostatin A treatment (Fig. 6E). These results show that transcriptional repression of the *Mlc3f* transgene in the right ventricle is not modified by overexpression of transcription factors normally present at the time when regionalisation is established.

Discussion

This study addresses the question of the maintenance, rather than the initial establishment, of transcriptional differences in cardiomyocytes of the right versus left ventricle. Expression of the *Mlc3f-nlacZ-2* transgene is restricted to cardiomyocytes of the right atrium and left ventricle of the developing heart from E10.5, and regionalisation is maintained throughout subsequent development (Kelly et al., 1998). We have used primary ventricular myocyte cultures to address the mechanisms by which this regional identity is maintained. Cardiomyocytes isolated from transgenic right and left ventricles maintain *Mlc3f* transgene expression

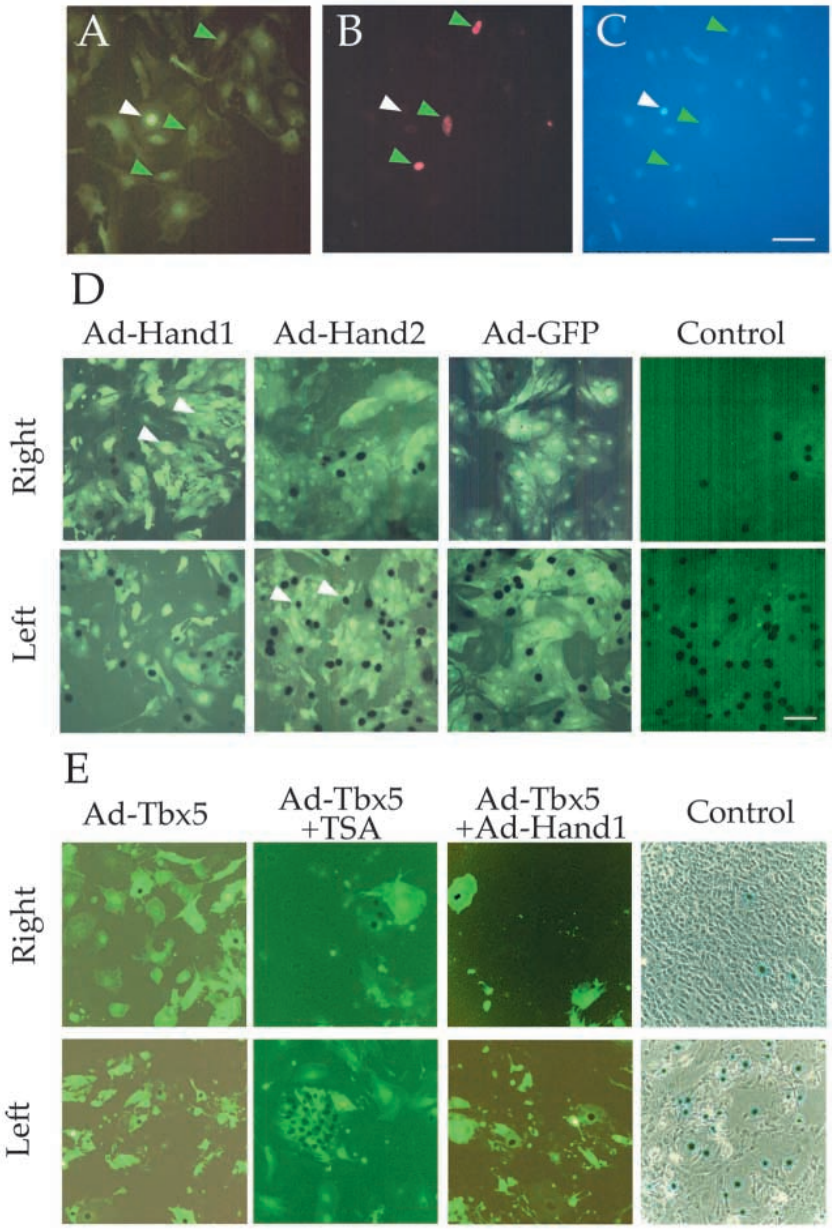

Fig. 6. Overexpression of *Hand1* and *Hand2* in primary ventricular cultures. (A) Anti-β-galactosidase immunocytochemistry of right ventricular transgenic cardiomyoytes after transfection with a *CMV-Hand1* expression vector showing a β-galactosidase-positive nucleus (white arrowhead). (B) Co-immunocytochemistry with an anti-myc tag antibody showing three positive nuclei (green arrowheads). (C) Hoechst staining reveals all nuclei in the field. (D) Overexpression of *Hand1* and *Hand2* under the control of a CMV promoter following adenoviral infection of primary cardiomyocytes prepared from left and right free ventricular walls of *Mlc3f-nlacZ-2* hearts. Cells were stained with X-gal (dark nuclei) and photographed under a fluorescent filter. All adenoviral-infected cells express green fluorescent protein. Despite a high degree of infection, *Mlc3f-nlacZ-2* transgene expression status is unaffected by overexpression of either *Hand* gene or of CMV-GFP alone. Control shows mock-infected X-gal-stained cells. White arrowheads indicate right ventricular cells infected with a *Hand1* encoding adenovirus that do not express the transgene and left ventricular cells infected with a *Hand2* encoding adenovirus that continue to express the transgene. (E) Overexpression of *Tbx5* under the control of a CMV promoter following adenoviral infection of right or left ventricular cardiomyocte cultures does not modify *Mlc3f-nlacZ-2* expression status in the presence or absence of trichostatin A (TSA), and co-infection with a *Hand1* encoding adenovirus. Cells were stained with X-gal (dark nuclei). Scale bars: 25 μm.

status in fetal primary cultures, whereas left and right cultures prepared from rats or nontransgenic mice express the same *Mlc3f* constructs equally after transient transfection. These results suggest that the establishment and maintenance of transgene expression status are achieved by different mechanisms and that developmental cell history plays a role in transcriptional regionalisation within the developing heart.

Titration of transfected DNA into nontransgenic primary cultures reveals that the loss of positional information after transient transfection is independent of plasmid DNA concentration. Thus, it is unlikely that differences in promoter copy number and titration of a right ventricular repressor after transient transfection contribute to the observed differences in activity. There are other differences between the transgene and the transiently introduced *Mlc3f* constructs: the former is integrated into the mouse genome, and it is uncoupled from bacterial vector sequences. In primary cardiomyocytes, which divide minimally, it is not possible to force integration of transiently introduced molecules. Chromosomal integration, independently of developmental cell history, may therefore contribute to the observed differences. Similarly, it cannot be excluded that the bacterial vector sequences on the transiently introduced *Mlc3f* constructs prevent transcriptional repression in *cis*. However, our results show that differential expression of the *Mlc3f-nlacZ-2* transgene in right versus left primary cultures is cell autonomous and independent of global methylation, trichostatin A-sensitive histone deactylation and treatment with hypertrophic agonists. Furthermore, the mechanism of transgene silencing in cardiomyocytes of the right ventricle is insensitive to the overexpression of *Hand1* or *Tbx5* – transcription factor-encoding genes that are normally expressed only in left ventricular cardiomyocytes, even in combination with trichostatin A treatment. These data suggest that although transgene expression status is established at the time of cardiac looping (Franco et al., 1997), a stage when elegant transplantation experiments have shown that an atrial versus ventricular phenotype is irreversibly established (Gruber et al., 1998), the transcriptional machinery that initiates transcriptional differences between right and left ventricles in the embryonic heart is no longer operative at late fetal stages. *Mlc3f* transgene expression status may similarly be irreversibly established during this early developmental time window.

Transcriptional repression of the *Mlc3f* transgene in right ventricular cardiomyocytes therefore appears to be maintained by mechanisms that are unable to silence molecules introduced after the closure of an early embryonic time-window during which expression status is established. These observations support a model by which epigenetic mechanisms underlie the maintenance of transgene silencing in the right ventricle. *Cis*-acting elements have been defined in *Drosophila* which convey the maintenance of reporter gene expression beyond the initial stages of embryogenesis when transcriptional status is established (Simon et al., 1993). These elements, termed ‘cellular memory modules’, interact with polycomb or trithorax group proteins to maintain a repressed or active state, and function as epigenetic switches (Lyko and Paro, 1999). Such elements are cell autonomous and independent of methylation. Furthermore, they are separable from the *cis*-acting elements that initially define transcriptional status and cannot be switched back at later developmental stages. Finally, although histone deacetylase activity may be involved in some aspects of polycomb-mediated silencing (Simon and Tamkun, 2002), in the case of the *Xenopus* polycomb homologue XPc1 transcriptional repression has been shown to be independent of histone deacetylase activity (Strouboulis et al., 1999). These features are in common with our observations concerning *Mlc3f* transgene repression and we suggest that the *Mlc3f* promoter contains a mammalian cellular memory module. Of particular interest is the recent finding that the mammalian polycomb-group homologue *Rae28* is essential for normal segmental development during cardiac morphogenesis and plays a role in the maintenance rather than in the initiation of expression of the gene encoding the essential cardiac transcription factor Nkx2.5 (Shirai et al., 2002). Histone methylation has recently been implicated in polycomb silencing in *Drosophila* (Czermin et al., 2002; Muller et al., 2002), and future experiments will address the role of histone methylation in *Mlc3f* transgene regionalisation.

Transgenesis permits the dissection and analysis of isolated regulatory elements that normally act together to coordinate endogenous gene expression. Six out of six transgenic lines containing 2 kb upstream of the *Mlc3f* transcriptional start site, with or without linked skeletal muscle enhancer sequences, are expressed in a left ventricular expression pattern (Kelly et al., 1998). The addition of enhancer sequences present at the endogenous locus modifies this pattern (Franco et al., 1997) and the endogenous gene, while showing left/right transcriptional regionalisation at E10.5, is subsequently downregulated in both ventricles (Kelly et al., 1998). Although the molecular basis for these observations remains unknown, an explanation can be given within the context of the above model. Additional regulatory sequences may modulate transcriptional status, possibly also acting through the proximal cellular memory module. Thus such an element could integrate information from a series of *cis*-acting regulatory sequences during early development and subsequently maintain transcriptional status, both cell autonomously and in a stably inherited manner.

In summary, our observations suggest that developmental cell history plays an important role in the maintenance of *Mlc3f* transgene silencing in the right ventricle. We propose that the regulatory sequences included in the transgene contain a cellular memory module, as defined in *Drosophila*, which enables the stable and heritable silencing of transgene transcription in right ventricular cardiomyocytes. Epigenetic mechanisms may therefore play a role in maintaining transcriptional diversity within the heart.

We are grateful to Diego Franco for helpful discussions and Stefano Schiaffino for advice on cardiomyocyte cultures. We thank Vincent Christoffels and Petra Habets for the ANF-luciferase reporter and VP16-Tbx2 construct. The laboratory of M.B. is supported by the Pasteur Institute and the CNRS, and grants from the ACI Integrative Biology Programme of the French Research Ministry and the EU Biomed programme (Grant Number 984004). R.G.K. and M.L. are INSERM research fellows. A.M. was supported by a Chateaubriand, Canadian Heart and Stroke Foundation fellowship.

References

Biben, C. and Harvey, R. P. (1997). Homeodomain factor Nkx2-5 controls left/right asymmetric expression of bHLH gene eHand during murine heart development. *Genes Dev.* **11**, 1357-1369.

Biben, C., Kirschbaum, B. J., Garner, I. and Buckingham, M. (1994). Novel muscle-specific enhancer sequences upstream of the cardiac actin gene. *Mol. Cell. Biol.* **14**, 3504-3513.

Bruneau, B. G., Logan, M., Davis, N., Levi, T., Tabin, C. J., Seidman, J. G. and Seidman, C. E. (1999). Chamber-specific cardiac expression of Tbx5 and heart defects in Holt-Oram syndrome. *Dev. Biol.* **211**, 100-108.

Bruneau, B. G., Nemer, G., Schmitt, J. P., Charron, F., Robitaille, L., Caron, S., Conner, D. A., Gessler, M., Nemer, M., Seidman, C. E. et al. (2001). A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell* **106**, 709-721.

Christoffels, V. M., Habets, P. E., Franco, D., Campione, M., de Jong, F., Lamers, W. H., Bao, Z. Z., Palmer, S., Biben, C., Harvey, R. P. et al. (2000). Chamber formation and morphogenesis in the developing mammalian heart. *Dev. Biol.* **223**, 266-278.

Cossu, G., Kelly, R., Tajbakhsh, S., Di Donna, S., Vivarelli, E., Buckingham, M. (1996). Activation of different myogenic pathways: myf-5 is induced by the neural tube and MyoD by the dorsal ectoderm in mouse paraxial mesoderm. *Development* **122**, 429-437.

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A. and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal polycomb sites. *Cell* **111**, 185-196.

Firulli, A. and Olson, E. (1997). Modular regulation of muscle gene transcription: a mechanism for muscle cell diversity. *Trends Genet.* **13**, 364-369.

Firulli, A. B., McFadden, D. G., Lin, Q., Srivastava, D. and Olson, E. N. (1998). Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. *Nat. Genet.* **18**, 266-270.

Franco, D., Kelly, R., Lamers, W., Buckingham, M. and Moorman, A. (1997). Regionalized transcriptional domains of myosin light chain 3f transgenes in the embryonic mouse heart: morphogenetic implications. *Dev. Biol.* **188**, 17-33.

Gruber, P. J., Kubalak, S. W. and Chien, K. R. (1998). Downregulation of atrial markers during cardiac chamber morphogenesis is irreversible in murine embryos. *Development* **125**, 4427-4438.

Habets, P. E., Moorman, A. F., Clout, D. E., van Roon, M. A., Lingbeek, M., van Lohuizen, M., Campione, M. and Christoffels, V. M. (2002). Cooperative action of Tbx2 and Nkx2.5 inhibits ANF expression in the atrioventricular canal: implications for cardiac chamber formation. *Genes Dev.* **16**, 1234-1246.

Hedlund, T., Meech, S. J., Li, S., Schaack, J., Hunger, S. P., Duke, R. C., DeGregori, J. and He, T. C. (1998). A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA* **95**, 13159-13164.

Kelly, R., Alonso, S., Tajbakhsh, S., Cossu, G. and Buckingham, M. (1995). Myosin light chain 3F regulatory sequences confer regionalized cardiac and skeletal muscle expression in transgenic mice. *J. Cell Biol.* **129**, 383-396.

Kelly, R., Zammit, P., Schneider, A., Alonso, S., Biben, C. and Buckingham, M. (1997). Embryonic and fetal myogenic programs act through separate enhancers at the MLC1F/3F locus. *Dev. Biol.* **187**, 183-199.

Kelly, R., Zammit, P., Mouly, V., Butler-Browne, G. and Buckingham, M. (1998). Dynamic left/right regionalisation of endogenous myosin light chain 3F transcripts in the developing mouse heart. *J. Mol. Cell. Cardiol.* **30**, 1067-1081.

Kelly, R., Zammit, P. and Buckingham, M. (1999). Cardiosensor mice and transcriptional subdomains of the vertebrate heart. *Trends Cardiovasc. Med.* **9**, 3-10.

Knowlton, K. U., Rockman, H. A., Itani, M., Vovan, A., Seidman, C. E. and Chien, K. R. (1995). Divergent pathways mediate the induction of ANF transgenes in neonatal and hypertrophic ventricular myocardium. *J. Clin. Invest.* **96**, 1311-1318.

Lin, Q., Schwarz, J., Bucana, C. and Olson, E. N. (1997). Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science* **276**, 1404-1407.

Lyko, F. and Paro, R. (1999). Chromosomal elements conferring epigenetic inheritance. *Bioessays* **21**, 824-832.

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L. and Harvey, R. P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. *Genes Dev.* **9**, 1654-1666.

MacNeill, C., French, R., Evans, T., Wessels, A. and Burch, J. B. (2000). Modular regulation of cGATA-5 gene expression in the developing heart and gut. *Dev. Biol.* **217**, 62-76.

McGrew, M. J., Bogdanova, N., Hasegawa, K., Hughes, S. H., Kitsis, R. N. and Rosenthal, N. (1996). Distinct gene expression patterns in skeletal and cardiac muscle are dependent on common regulatory sequences in the MLC1/3 locus. *Mol. Cell. Biol.* **16**, 4524-4534.

Muller, J., Hart, C. M., Francis, N. J., Vargas, M. L., Sengupta, A., Wild, B., Miller, E. L., O'Connor, M. B., Kingston, R. E. and Simon, J. A. (2002). Histone methyltransferase activity of a Drosophila polycomb group repressor complex. *Cell* **111**, 197-208.

Newell-Price, J., Clark, A. J. and King, P. (2000). DNA methylation and silencing of gene expression. *Trends Endocrinol. Metab.* **11**, 142-148.

Riley, P., Anson-Cartwright, L. and Cross, J. C. (1998). The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. *Nat. Genet.* **18**, 271-275.

Sadoshima, J., Xu, Y., Slayter, H. S., Izumo, S. (1993). Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell* **75**, 977-984.

Sanes, J. R., Rubenstein, J. L. R. and Nicolas, J.-F. (1986). Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *EMBO J.* **5**, 3133-3142.

Schwartz, R. J. and Olson, E. N. (1999). Building the heart piece by piece: modularity of cis-elements regulating Nkx2-5 transcription. *Development* **126**, 4187-4192.

Shirai, M., Osugi, T., Koga, H., Kaji, Y., Takimoto, E., Komuro, I., Hara, J., Miwa, T., Yamauchi-Takihara, K. and Takihara, Y. (2002). The Polycomb-group gene Rae28 sustains Nkx2.5/Csx expression and is essential for cardiac morphogenesis. *J. Clin. Invest.* **110**, 177-184.

Simon, J., Chiang, A., Bender, W., Shimell, M. J., O'Connor, M. (1993). Elements of the Drosophila bithorax complex that mediate repression by Polycomb group products. *Dev. Biol.* **158**, 131-144.

Simon, J. A. and Tamkun, J. W. (2002). Programming off and on states in chromatin: mechanisms of Polycomb and trithorax group complexes. *Curr. Opin. Genet. Dev.* **12**, 210-218.

Srivastava, D. (1999). HAND proteins: molecular mediators of cardiac development and congenital heart disease. *Trends Cardiovasc. Med.* **9**, 11-18.

Srivastava, D., Thomas, T., Lin, Q., Kirby, M. L., Brown, D. and Olson, E. N. (1997). Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor dHAND. *Nat. Genet.* **16**, 154-160.

Strouboulis, J., Damjanovski, S., Vermaak, D., Meric, F. and Wolffe, A. P. (1999). Transcriptional repression by XPc1, a new Polycomb homolog in Xenopus laevis embryos, is independent of histone deacetylase. *Mol. Cell. Biol.* **19**, 3958-3968.

Vikstrom, K. L., Bohlmeyer, T., Factor, S. M. and Leinwand, L. A. (1998). Hypertrophy, pathology, and molecular markers of cardiac pathogenesis. *Circ. Res.* **82**, 773-778.

Wade, P. A. (2001). Transcriptional control at regulatory checkpoints by histone deacetylases: molecular connections between cancer and chromatin. *Hum. Mol. Genet.* **10**, 693-698.

Wang, G. F., Nikovits, W., Jr, Bao, Z. Z. and Stockdale, F. E. (2001). Irx4 forms an inhibitory complex with the vitamin D and retinoic X receptors to regulate cardiac chamber-specific slow MyHC3 expression. *J. Biol. Chem.* **276**, 28835-28841.

Zammit, P. S., Kelly, R. G., Franco, D., Brown, N., Moorman, A. F. M. and Buckingham, M. E. (2000). Suppression of atrial myosin gene expression occurs independently in the left and right ventricles of the developing mouse heart. *Dev. Dyn.* **217**, 75-85.