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

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Summary

The molecular mechanisms that establish and maintain transcriptional differences between cardiomyocytes in the left and right ventricular chambers are unknown. 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 atroventricular 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 (Furulli 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 deacytlation. 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 CO2. Femoral vein blood was removed and placed in 6 cm dishes at a density of 7-8×10^5 per plate in plating medium containing newborn calf serum and grown at 37°C in 5%CO2. Medium was replaced after 18 hours, at which stage beating cardiomyocytes were observed.

In co-culture assays, 10^6 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 (10^3 to 10^6) 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 0.5μg of a reporter gene containing the Rous Sarcoma virus long terminal repeat linked to the luciferase gene (RSVlac) 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 HpaII or MspI 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 Cila-BamHI 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 ATAGAATGCGCCGAGATGAGCTACGCAC and Hand1Rev GCTCTAGACTAGTTTAGCTCCAGCGCCCAG were used. For the Hand2 fragment (+8/+1672) primers Hand2Forw ATAGAATGCGCCGCATAGCTGCTGTGGGGGCGTCTCCACG and Hand2Rev GCTCTAGCTAGACTACTCTGTGGACTCCAGGGCC were used. For the Tbx5 fragment (+193/+1749) primers Tbx5Forw GGAATTCGCGGCCGCGACTGCGCAGAAGAATGCGGCCGAGC and Tbx5Rev GCTCTAGATATTCTCACTCCACTCCACTCCATGAGTCGAGCTGGC 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 recombiant 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. 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 20x 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.

**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-2E 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 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 Mlc1f3f 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
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 nontransgenic right ventricular cardiomyocytes, 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 HpaII/MspI sites. Genomic DNA isolated from right and left ventricles, in addition to right and left atria, was digested with HpaII (methylation sensitive) or MspI (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 HpaII (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 acetyl residues from histones leads to a closed chromatin configuration and transcriptional silencing. To investigate whether histone deacetylation 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. 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.](image-url)
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).

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.
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 deacetylation 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.

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