In Vitro Chamber Specification during Embryonic Stem Cell Cardiogenesis

EXPRESSION OF THE VENTRICULAR MYOSIN LIGHT CHAIN-2 GENE IS INDEPENDENT OF HEART TUBE FORMATION*

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The molecular cues that control patterning of the heart tube during early cardiogenesis are largely unknown. The present study has explored the embryonic stem (ES) cell differentiation system to determine if this in vitro model could be useful in studying the process of regional specification of cardiac muscle cells at the earliest possible stages. As assessed by polymerase chain reaction, ribonuclease protection, in situ hybridization, and immunohistochemical analyses, ES cell differentiation into embryoid bodies is characterized by the transcriptional and translational activation of the ventricular regulatory (phosphorylatable) myosin light chain gene. Demonstrating that ventricular specification occurs during ES cell cardiogenesis. The finding of a ventricular-specific marker in an in vitro system in the absence of an intact heart tube provides evidence for cardiac regional specification independent of positional cues or physiologic stimuli. The temporal expression of the myogenic regulatory factors, myogenin and MyoD, suggests activation of the skeletal muscle program following cardiac myogenesis in vitro, indicating temporal fidelity to the progression of in vitro myogenesis. These data establish the mouse embryonic stem cell system as a model for cardiac chamber specification and suggest a promising approach in the study of regional specification in genetically engineered cardiac muscle cells.

A critical step during mammalian cardiogenesis is the formation of distinct atrial and ventricular chambers that will eventually drive the pulmonary and systemic circulations in the mature heart. The acquisition of the divergent morphological, electrophysiological, and biochemical properties of the specialized cell types that constitute the different cardiac chambers is largely due to the activation of regional specific programs of muscle gene expression during cardiac development. Although basic region helix-loop-helix (bHLH) myogenic regulators, MyoD and related family members, for skeletal muscle cell lineages have been extensively studied (for review, see Olson (1993)), these genes are not expressed in cardiac muscle, and there are currently no known cardiac-specific bHLH proteins. In fact, increasing evidence suggests that divergent programs may control the different muscle specificities of a single gene that is expressed in both cardiac and skeletal muscle (Lee et al., 1992). Although a small number of transcriptional factors that may participate in regulating cardiac muscle genes have been identified (Pollock and Treisman, 1991; Farrance et al., 1992; Yu et al., 1992; Adolf et al., 1993; Zhu et al., 1993), a clear view of how cardiac specificity is conferred is lacking. Accordingly, the molecular and positional cues that control patterning of the heart tube or regional specification of muscle cells during early embryogenesis are poorly understood.

Our previous studies have established the gene that encodes the ventricular isoform of the cardiac regulatory myosin light chain (MLC-2V) as a valuable model for the elucidation of molecular mechanisms that underlie the regulation of muscle gene expression during cardiac growth and development (Chien et al., 1992, 1993). Within the normal adult mammalian myocardium, this gene is expressed exclusively in the ventricular chamber, with negligible expression in the atrium. In contrast to other genes, which are co-expressed in all cardiac chambers throughout the early looped heart and acquire regional specificity relatively late during the course of cardiogenesis (post-septation) or postnatal period (Lyons et al., 1990), the MLC-2V gene displays positional specification to the ventricular segment of the primitive linear heart tube, preceding cardiac septation and the development of distinct cardiac chambers (O'Brien et al., 1993).

The lack of continuous cell lines that can mimic the tran...
sition from a pluripotent stem cell to specific cardiac muscle cell lineages (atrial, ventricular, conduction system) has represented a major impediment for coupling molecular and genetic approaches to studying the sequential steps toward differentiation. Pluripotent ES cells, derived from the inner cell mass of mouse blastocysts, can be maintained for many generations in the undifferentiated state in culture, retaining their embryonic phenotype (Evans and Kaufman, 1981; Martin, 1981). When ES cells are removed from the differentiated inflammatory influence of feeder cells or their equivalent, the cells spontaneously give rise to structures resembling those found in an embryo. The "embryo-like" structures or embryoid bodies express markers of endodermal, ectodermal, and mesodermal differentiation and have been used as in vitro model systems to study hematopoiesis, vasculogenesis, and angiogenesis (Risau et al., 1988; Lindenbaum and Grosveld, 1990; Schmitt et al., 1991; Wiles and Keller, 1991; Wang et al., 1992; Keller et al., 1993). In addition, embryoid bodies express a number of muscle markers, including myosin heavy chain and tropomyosin (Robbins et al., 1990, 1992; Wieczorek et al., 1990; Sanchez et al., 1991; Muthuchamy et al., 1993), suggesting that this system can serve as a model to study early aspects of cardiac differentiation.

To determine if embryonic stem cells could be useful in studying the process of regional cardiac muscle cell specification in vitro, the current study has utilized the cardiac MLC-2V gene as a genetic marker of the process of ventricular specification. A combination approaches based on polymerase chain reaction (PCR)/ribonuclease (RNase) protection, and in situ hybridization documents ventricular specification during ES cell cardiogenesis. The results demonstrate that the activation of myogenic determination/differentiation genes (myogenin and MyoD) lags behind cardiac myogenesis, thereby displaying temporal fidelity with the onset of expression of these muscle markers during normal embryogenesis. Accordingly, these studies suggest that genetic manipulation of ES cells will now allow genetic approaches to study the complex process of cardiac chamber specification. In addition, these results in an in vitro system suggest that the activation of ventricular MLC-2 gene expression is not dependent upon positional cues that arise from the formation of an intact heart tube, as occurs during cardiogenesis in vivo.

**EXPERIMENTAL PROCEDURES**

**Cell Culture.—**The mouse blastocyst-derived D3 embryonic stem cell line (Doetschman et al., 1986) was generously provided by Dr. T. C. Doetschman (University of Cincinnati). ES cells were propagated in high glucose Dulbecco's modified Eagle medium (Life Technologies, Inc.) supplemented with 15% heat-inactivated fetal calf serum (Sigma), 2 mM L-glutamine and 0.1 mM β-mercaptoethanol (Sigma). Cells were maintained in the undifferentiated state either by culture on confluent feeder layers of mitomycin C-treated primary mouse embryonic fibroblasts or by the addition of purified recombinant mouse leukemia inhibitory factor (LIF; Esgro, Life Technologies, Inc.) at 1,000 units/ml to the culture media. Under these conditions, the majority of the ES cells (exceeding 95%) displayed an undifferentiated phenotype, as assessed by visual inspection under phase-contrast microscopy. Cells were maintained at 37 °C in a humidified atmosphere of 10% CO2 in air. Monolayers were passaged by trypsinization at 70-80% confluence.

**RNA Isolation and cDNA Synthesis—**Total cellular RNA was isolated from feeder-layer fibroblasts, ES cells, or ES cell-derived embryoid bodies by the RNAzol method (Cinna Biotech). First-strand cDNA synthesis was performed using 5 µg of total RNA, Moloney murine leukemia virus RNase H+ reverse transcriptase, and 100 ng of random hexamers, according to the manufacturer's recommendations (Life Technologies, Inc.) in a final reaction volume of 40 µl. Following termination of the first-strand synthesis reaction, the samples were incubated with RNase H at 37 °C for 20 min. The same first-strand preparation was used for analyzing each of the gene products by PCR.

**Polymerase Chain Reaction and Analysis of Amplified Products—**PCR amplifications were performed with 5% of the first-strand reaction, 2.5 units of Taq polymerase (Life Technologies, Inc.), and 50 pmol of the appropriate primers in a reaction volume of 50 µl. The reactions were carried out in an automated thermal cycler (Eriocmp). The amplification sequence consisted of an initial denaturation at 94 °C for 4 min, followed by 5 cycles of denaturation at 94 °C for 45 s, annealing at 55-65 °C for 1 min (depending upon the melting temperature of the oligonucleotide primers), and extension at 72 °C for 2 min, and 30 cycles of 94 °C for 45 s, 45-55 °C for 1 min, and 72 °C for 2 min. A final extension at 72 °C for 10 min was performed. The authenticity of the amplified product was determined by size analysis (15 µl of the reaction was separated on 2% agarose gel).

**Ribonuclease Protection Studies—**RNase protection assays were performed as previously described (Zhu et al., 1991). For the assessment of the expression of the mouse ventricular regulatory light chain gene, a 460-bp probe (406-bp protected fragment) was generated by Xbal restriction of a plasmid containing the MLC-2V cDNA (mMLC-2V-510). To assay levels of atrial natriuretic factor (ANF) mRNA, a rat ANF cDNA representing the entire coding region cloned into the PstI site of pGEM-7) was used (Knoy et al., 1991). Digestion of the plasmid with XhoI and transcription with T7 RNA polymerase generated a 141-bp probe (95-bp protected hybrid with test RNA). Mouse cardiac atrial (MLC-1A) and ventricular light chain (MLC-IV) riboprobes were derived by EcoRI restriction of appropriate plasmids subcloned into the Bluescribe vector and transcription with T7 polymerase (Barton et al., 1988; Lyons et al., 1990). The RNA probes were labeled with [32P]CTP and purified on an 8 M urea, 6% polyacrylamide gel. Five to 15 µg of total RNA was hybridized with 50,000-150,000 cpm of the respective purified probe at 45 °C overnight. The unprotected RNA was subsequently digested with RNase A at 25 °C for 60 min. The reaction was terminated with SDS and proteinase K, and the reaction mixture was phenol-extracted and ethanol precipitated. The RNA was dissolved in water and degraded by gel electrophoresis on a denaturing polyacrylamide/urea gel. The gel was dried, and autoradiography was performed for various time periods until signal was detected.

**In Situ Analyses of Embryoid Bodies—**In situ hybridizations were performed essentially as described by Iffrig et al. (1990). Briefly, embryoid bodies were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated, and infiltrated with
paraffin. Serial sections (5-7 μm in thickness) were mounted on gelatinized slides, deparaffinized in xylene, and rehydrated. Following proteinase K digestion, the sections were post-fixed, treated with triethanolamine/acetic anhydride, washed, and dehydrated. In order to distinguish between transcripts within the same multigene family, riboprobes were derived from the 3' noncoding untranslated regions (UTR) of the sequences of interest. The following probes were used: 3' UTR of the mouse MLC-1A mRNA (Barton et al., 1988; Lyons et al., 1990), 3' UTR of the mouse MLC-1V mRNA (Lyons et al., 1990), 3' UTR of the rat ventricular myosin heavy chain (MHC β) mRNA (Mishdavi et al., 1982; Lyons et al., 1990), 3' end of the mouse atrial myosin heavy chain (MHC α) mRNA (Weydart et al., 1985; Lyons et al., 1990), 3' end of the mouse MLC-2V mRNA (Lee et al., 1992), and 3' end of the rat ANF mRNA (Knowlton et al., 1991). Sections were hybridized overnight at 52 °C with 50,000-75,000 cpm/ml 32P-labeled cRNA probe. The tissues were subjected to stringent washes (2 X SSC (1 X SSC is 0.15 M NaCl, 0.015 M sodium citrate solution), 50% formamide, 10 mM dithiothreitol at 65 °C) and RNase A treatment (20 μg/ml) at 37 °C for 30 min. The slides were dehydrated, immersed in photographic emulsion, and exposed for a week in light-tight boxes. Following photographic development, slides were analyzed using both light and dark field optics.

Immunohistochemistry—Single cell suspensions were prepared from embryonic bodies using trypsin-EDTA (Life Technologies, Inc.), plated in plastic chamber slides (Nunc), and maintained in growth media (as for embryoid bodies) for a period of 48 h. Contractile activity was evident in single cells or groups of cells following their attachment to the tissue culture substrate. Indirect immunofluorescence experiments were performed according to a previously described method (Shubetta et al., 1990). Briefly, fixation was carried out in 3% paraformaldehyde and following incubation in 50 mm NH4Cl for 10 min, the cells were permeabilized with 0.2% Triton X-100 in PBS. Following a step to block nonspecific binding sites (1% bovine serum albumin, for 10 min), the monolayers were incubated at 37 °C for 60 min with the appropriate primary antibody, polyclonal rabbit antibody against the ventricular myosin light chain-2 (Iwaki et al., 1990) and/or monoclonal mouse antibody against atrial natriuretic factor (generously provided by Dr. C. C. Glembocki, San Diego State University). Secondary antibodies were applied as controls: goat anti-rabbit IgG conjugated to fluorescein (Jackson ImmunoResearch Laboratories, Inc.) to recognize MLC-2V and biotinylated sheep anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories, Inc.), followed by Texas Red-conjugated streptavidin (Amerham Corp.) for ANF. The slides were rinsed in PBS, mounted with propyl gallate, and examined with both a Nikon microscope equipped with epifluorescence optics and a Bio-Rad 4000 series laser scanning confocal microscope.

RESULTS

ES Cell Culture and Embryoid Body Differentiation—In order to inhibit ES cell differentiation and to promote proliferation and retention of a normal diploid karyotype, defined culture conditions are required. One approach is to seed ES cells over a layer of fibroblasts previously treated with irradiation or drugs to inhibit further cell division (Doetschman et al., 1985). An alternative culture method that obviates the need for feeder cells is the maintenance of ES monolayers in the presence of leukemia inhibitory factor, known to inhibit ES cell differentiation (for review, see Metcalf, 1991). We compared gene expression in both ES cells and derived embryoid bodies grown under these two culture conditions and were not able to detect significant differences in the temporal expression of muscle genes or the level of expression of these markers (data not shown).

Significant heterogeneity in the size and morphology of the cell aggregates is apparent throughout the course of ES cell differentiation in suspension cultures. In consideration of the potential influence of this variability on the onset and extent of gene transcription, culture conditions were manipulated to optimize the homogeneity of the starting cell aggregates and result in a more synchronous differentiation system. This was accomplished by ensuring that the differentiation program was initiated in fully dissociated ES cell suspensions (for details, see "Experimental Procedures"). This technique is not only known to improve the reproducibility of the timing of gene expression, it also results in higher overall levels of expression (Lindenbaum and Grosveld, 1990). These conditions gave rise to cell aggregates, which typically displayed spontaneous foci of rhythmic contractions by day 8 in suspension culture.

Amplification of Gene Products Expressed in ES Cells and Embryoid Bodies—The kinetics of induction of a profile of endogenous muscle genes during ES cell differentiation was investigated using the reverse transcriptase-dependent polymerase chain reaction (RT-PCR). In order to perform a semi-quantitative analytical comparison between the amplified samples for a particular transcript on the same time course, cDNA samples were adjusted to yield a relatively equal amplification of a ubiquitously expressed standard, β-tubulin, prior to their analyses (Fig. 1A). This was performed by quantitation of incorporated radiolabeled CTP into the β-tubulin standard and ethidium bromide staining following calibration of cDNA samples. Oligonucleotide-specific primers designed to assess mRNA levels throughout embryoid body development generally included the 3' UTR and, when possible, spanned at least one intron of the associated gene (Table I). The amplification reactions were performed in parallel with appropriate controls (excluding RNA template or reverse transcriptase in the cDNA synthesis reaction) to discriminate that the amplified fragments were derived from mRNA and not from genomic DNA or other contaminants, which could potentially influence the PCR results. The temporal expression of the gene products of interest was determined in pools of embryoid bodies, each different time point representing additional days in suspension culture. The time point at which a particular transcript undergoes significant expression would suggest when that gene begins to play a role in the differentiation process. Representative amplifications of four to six independent RNA series are shown.

Muscle Gene Expression during ES Cell Differentiation—To determine the kinetics of muscle development during the differentiation of ES cells into embryoid bodies, the expression pattern of a subset of muscle mRNA sequences specific to be developmentally regulated in the mouse heart was assessed. Analysis of myosin heavy chain transcript expression reveals the up-regulation of both α- and β-cardiac isoforms during embryoid body development (Fig. 1B). β-Cardiac MHC transcripts were detected from day 6 of differentiation and continued to be evident throughout the developmental period examined (up to 21 days in suspension culture). α-Cardiac MHC gene products were present from day 9 through day 21 of differentiation. Atrial alkali myosin light chain (MLC-1A) transcripts were initially evident between days 6 and 9 of embryoid body differentiation, with expression continuing as differentiation proceeded (Fig. 1B). The time course of MLC-1V gene expression demonstrated earlier transcriptional activation. A faint signal was detected in fibroblasts and embryonic stem cells and during the first few days of suspension cultures. A definite increase in the amplified mRNA signal was seen in embryoid bodies between days 6 and 20 of differentiation (Fig. 1B). This observation was confirmed in five distinct series, including those derived from ES cells maintained either on a layer of fibroblasts or on LIF. ANF mRNA expression was also characterized in the developing embryoid bodies. ANF message was initially detected

2 W. C. Miller-Hance, unpublished observations.
Expression of the ANF gene was also supported by corresponding RNase protection studies (Fig. 2B). Thus, these analyses parallel the PCR data regarding the temporal expression of muscle gene transcripts during ES cell differentiation, documenting the appropriate, stage-specific pattern of expression of these genes in the differentiation cultures. A combined summary of the PCR and RNase protection analyses is presented in Table II.

Expression of the MLC-2V Gene, a Molecular Marker for Ventricular Specification, during in Vitro Cardiogenesis—Recent studies in our laboratory regarding ventricular MLC-2 mRNA expression during murine cardiogenesis have documented early positional specification of this gene in the primitive heart tube exclusively in regions destined to become ventricle, providing evidence for regional specification of the ventricular muscle gene program at the earliest stages of mammalian cardiogenesis (O'Brien et al., 1993). In view of this property, which characterizes the cardiac MLC-2V gene as a genetic marker for ventricular specification, we wished to address whether in vitro cardiogenesis is also highlighted by the transcriptional activation of this muscle sequence. The data shown in Fig. 1C document the expression of ventricular regulatory light chain gene transcripts throughout the differentiation of ES cells into embryoid bodies. Amplification of MLC-2V mRNA yields a low level of gene expression initially identified between days 8 and 10 of embryoid body differentiation, concomitant with the appearance of spontaneous contractile activity in the cultures by microscopic observation. MLC-2V message continues to be evident with advancing days in culture.

RNA protection studies were also conducted to validate the MLC-2V amplification results. These analyses were performed using an antisense mouse cardiac MLC-2 riboprobe encompassing nearly the entire coding region. A protected fragment representing RNase-resistant hybrids is initially detected between 8 and 10 days of embryoid body differentiation (Fig. 2B). These results confirm the RT-PCR findings regarding the temporal expression of the MLC-2V gene in this model system.

Expression of Helix-Loop-Helix Transcription Factors in Developing Embryoid Bodies—During the last decade significant progress has been made in the field of muscle developmental biology by the identification and characterization of a gene family encoding master transcriptional factors that are able to mediate the myogenic programming of mesodermal cells (for review, see Weintraub et al. (1991) and Olson (1993)). The basic helix-loop-helix family of myogenic regulators that control establishment of the differentiated muscle phenotype includes myf-5, myogenin, MyoD, and MRF-4/herculin/myf-6. However, despite the shared expression of numerous contractile protein genes by striated muscles common to both skeletal and cardiac types, particularly throughout early development, none of the previously characterized myogenic regulatory genes has been identified in the embryonic, neonatal, or adult heart. Since the activation of the skeletal muscle program is characterized by the up-regulation of these sequences, we were interested in defining the onset of skeletal myogenesis in this in vitro system as identified by the expression of three myogenic regulatory factors, myf-5, myogenin, and MyoD. These data would also define whether the transcriptional activation of other muscle genes examined during ES cell differentiation was associated with skeletal versus cardiac myogenesis. PCR analysis documented the expression of myf-5 transcripts in ES cells and throughout their differentiation (Fig. 3). An increase in myf-5 mRNA
levels was seen with additional days in culture (days 12-20). Myogenin and MyoD transcripts were evident during days 16-20 of differentiation (Fig. 3). We were also interested in assessing the expression of the inhibitory helix-loop-helix protein, Id, in this in vitro system. Id is expressed in a wide variety of proliferating cell types, and mRNA levels appear to be high in proliferating myoblasts in culture with down-regulation occurring during differentiation (Benezra et al., 1990). The present study demonstrates high levels of Id-1 expression in undifferentiated ES cells, which persist throughout embryoid body development (Fig. 3). No significant changes in Id-1 mRNA signal were observed as differentiation progressed in the embryoid bodies.

Spatial Pattern of Muscle Gene Expression in Beating Embryoid Bodies—Developing embryoid bodies exhibit foci of spontaneous rhythmic contractile activity, the extent of this activity being primarily influenced by culture conditions. Light microscopy examination usually reveals either single areas or, in some cases, multiple regions of contraction within a single embryoid body. To determine the spatial pattern of distribution of muscle gene transcripts within beating embryoid bodies and to address whether the same pattern of cardiac-specific gene expression observed in vitro is recapitulated in vivo is critical. Cardiac-specific antisense probes were performed on serial sections of embryoid bodies at various time points after differentiation was initiated. Figs. 4 and 5 display two different embryoid bodies cultured for 21 days and hybridized with riboprobes to myosin light chains, myosin heavy chains, and atrial natriuretic factor

### TABLE I

| Gene   | 5' primer sequence | 3' primer sequence | Size of amplified product |
|--------|--------------------|--------------------|--------------------------|
| β-Tubulin | 5'-TCACATGCTGCTGAATTCG-3' | 3'-CGTCAAAATGGCCGATACAG-5' | 317 bp |
| MHC α  | 5'-CTGCTGAGGCCGATTCG-3' | 3'-GGAACTACGCAGCGTAGAAG-5' | 302 bp |
| MHC β  | 5'-CTGACGGCTCGGACGGG-3' | 3'-GTCGAGCTCTAAGATATT-5' | 205 bp |
| MLC-1A | 5'-GTCGTCAGCCGCAGGCTTGA-3' | 3'-CTTCTCAGCAAGGGTCAGA-5' | 131 bp |
| MLC-1V | 5'-GCAAAAGGACGCGAGAGAAG-3' | 3'-CTGACTCGGGACTTGGTGTC-5' | 86 bp |
| ANF    | 5'-CCTCTGCGACGGAGGCTG-3' | 3'-CTTGAACCTGTCCAACCACAACCG-5' | 400 bp |
| MLC-2V | 5'-GCCCAAGAGGCGATAGAAGG-3' | 3'-CTCTCATCTAAGTTGTCG-5' | 308 bp |
| myf-5  | 5'-CCACACGCACTCTGCTTCCTG-3' | 3'-AGTCTGCTACTCCTCGTGCA-5' | 333 bp |
| Myogenin | 5'-AGCTCTCTTACACAGGAGAG-3' | 3'-TCTTCAAGTCGAGTCGTCGCA-5' | 425 bp |
| MyoD   | 5'-CAATCGAGCAGCTGAGTCG-3' | 3'-CAATTAGTGGGCGCACAAA-5' | 562 bp |

### FIG. 2. Muscle gene expression during ES cell differentiation as assessed by RNase protection analyses. Total RNA was derived from fibroblasts (Fb), undifferentiated ES cells (ES), and embryoid bodies at various time points (D) and analyzed by RT-PCR and RNase protection analyses. **summary of PCR amplification and RNase protection analyses**

| Gene   | Fb | ES | D3 | D6 | D9 | D12 | D16 | D20 |
|--------|----|----|----|----|----|-----|-----|-----|
| MHC α  | -  | -  | ++ | ++ | ++ | ++  | ++  | ++  |
| MHC β  | -  | -  | +/−| +/−| +/−| +/− | +/− | +/− |
| MLC-1A | -  | -  | +/−| +/−| +/−| +/− | +/− | +/− |
| MLC-1V | +/−| +/−| +/−| +/−| +/−| +/− | +/− | +/− |
| ANF    | -  | -  | +/−| +/−| +/−| +/− | +/− | +/− |
| myf-5  | -  | -  | +/−| +/−| +/−| +/− | +/− | +/− |
| Myogenin | -  | -  | -  | -  | -  | -   | -   | -   |
| MyoD   | -  | -  | -  | -  | -  | -   | -   | -   |
| Id-1   | ++ | ++ | ++ | ++ | ++ | ++  | ++  | ++  |

### FIG. 3. Expression of myogenic regulatory factors during ES cell differentiation as assessed by RT-PCR. Analysis of myf-5, myogenin, MyoD, and Id-1 PCR products following 30 cycles of amplification, agarose gel electrophoresis, and ethidium bromide staining. The particular gene detected in any given amplification is indicated. Fb, fibroblasts; ES, undifferentiated ES cells; D, number of days of embryoid body differentiation in suspension cultures.
mRNAs in individual sections. In Fig. 4, neither MLC-2V nor ANF mRNAs are detected in cardiac myocytes (panels C and D) where MLC-1V, MLC-1A, and MHC α are expressed (panels B, E, and F, respectively). In Fig. 5, MLC-2V and ANF gene transcripts are detected in a subset of the cardiac myocytes expressing MHC α, MHC β, and MLC-1V mRNAs. These findings are consistent with in situ hybridization studies in vivo, which document that MLC-2V and ANF mRNAs are localized in a subpopulation of myocytes in the developing heart tube (O'Brien et al., 1993). In contrast, MHC α, MLC-1A, and MLC-1V transcripts are detected in most if not all cardiac myocytes from days 8–11 post coitum (Lyons et al., 1990).

**Muscle Gene Expression in Embryoid Body-derived Cardiac Muscle Cells**—To investigate the expression of muscle gene translational products in single cells derived from embryoid bodies, immunofluorescence studies were performed following the dispersion of beating cell aggregates. The single cell level analysis using antibodies directed against the regulatory ventricular light chain and atrial natriuretic factor revealed cells that are positive for both MLC-2V and ANF expression. The staining pattern of the MLC-2V antibody, identified striations characteristic of the sarcomeric structures, confirming the muscle nature of the differentiated cells (Fig. 6A). The typical perinuclear location of ANF gene expression in the same field of cells is also shown (Fig. 6B). This finding provides evidence that embryoid body-derived muscle cells express genes that are restricted to specific cardiac cell types.

Further inspection of the stained cells reveals at least four cellular subpopulations, as assessed by distinct immunofluorescence phenotypes. These include cells that do not recognize either MLC-2V or ANF antibodies, cells that express both gene products, and cells that only express either MLC-2V or ANF. Three of these cellular phenotypes are depicted in Fig. 6 (panels A, B, and E). Although the precise nature of these various cells is unclear, it could be speculated that the ANF-positive, MLC-2V-negative cells may represent cardiac muscle progenitors or ventricular cells at an early stage in the specification program and not yet expressing the regulatory ventricular myosin light chain. More interestingly, we suggest that these cells may be atrial cells, which characteristically express ANF at high levels and negligible amounts of ventricular MLC-2. The identification and characterization of additional atrial-specific genetic markers should provide further information regarding the nature of this particular cellular subtype.

**DISCUSSION**

The Temporal Activation of Muscle Genes during in Vitro ES Cell Myogenesis Mimics Embryonic Myogenesis—Despite significant recent accomplishments in the area of myocardial growth and developmental regulation, defining the molecular basis underlying these events continues to present a fundamental challenge in the study of the developmental biology and molecular genetics of the cardiovascular system (Chien, 1993). The embryonic stem cell system provides a promising approach in the investigation of gene regulation during cardiac growth and development. Previous studies have demonstrated that hematopoietic differentiation in ES cell-derived embryoid bodies follows a well defined temporal pattern of gene expression, similar to that involved in the establishment of the hematopoietic system in the normal embryo (Lindenbaum and Grosfeld, 1990; Schmitt et al., 1991; Keller et al., 1993). It has also been shown that embryoid bodies transcribe muscle genes (myosin heavy chain and tropomyosin isoforms) in an appropriate tissue- and developmental stage-specific pattern (Robbins et al., 1990; Muthuchamy et al., 1993), suggesting that developmental stages in ES cell culture reflect normal embryonic transitions. Likewise, the present study has exploited the features of this system that mimic in vivo embryogenesis to demonstrate that the developmental gene program activated during establishment and differentiation of muscle lineages in vitro parallels the sequence of events occurring in the developing mouse embryo. Myosin heavy chain genes (α and β), alkali myosin light chains (MLC-1A and MLC-1V), and atrial natriuretic factor transcripts are each expressed in an appropriate, stage-specific pattern, with negligible expression of these markers in undifferentiated ES cells and sequential transcriptional activation ensuing upon
FIG. 6. Indirect immunofluorescence analysis of MLC-2V and ANF gene expression in single cells derived from pools of beating embryoid bodies. Single cells were obtained from the dispersion of pools of rhythmically contracting embryoid bodies at day 11 of differentiation, as described under "Experimental Procedures." The cells were fixed and analyzed by indirect immunofluorescence using antibodies directed against MLC-2V and ANF proteins. Panels A and B, low power magnification of a group of cells within the same field that recognize the MLC-2V (A, green signal) and ANF (B, red signal) antibodies. The arrows in panels A and B point to a single cell that recognizes the MLC-2V antibody but not ANF (MLC-2V-positive/ANF-negative). Panels C and D, high power magnification (×400) of a different group of cells that express MLC-2V (panel C) and ANF (panel D) proteins within the same field. Panel E, two additional cellular subtypes as identified by MLC-2V/ANF double staining are shown (same field of cells as panels C and D). The arrow indicates a cell positive for both MLC-2V and ANF, whereas the arrowhead points to a cell that expresses exclusively the ANF protein (MLC-2V-negative/ANF-positive).
differentiation. The kinetic analysis of the expression of myogenic regulatory genes during ES cell differentiation demarcates the operative ventricular myogenic expression of myf-5 and MyoD transcripts at relatively late stages of embryoid body differentiation (days 16–20), well beyond the initiation of rhythmic contractile activity in the suspension cultures. Although myf-5 gene expression is evident at earlier time points, this may be consistent with recent data suggesting that myf-5 up-regulation may not be unique to the establishment of the muscle lineage, since products of a transgene linked to the myf-5 control region have also been identified in the developing murine nervous system. In myf-5-expressing embryoid bodies, neurofilament mRNA and protein have also been detected, suggesting that myf-5 transcriptional activation during early stages of embryoid body development may be the result of concomitant activation of the neural program. The temporal pattern of expression of the myogenic factors, myogenin and MyoD, provides evidence for activation of the skeletal muscle program subsequent to the development of beating myocardium. This progression of in vitro myogenesis documents that the ES cell differentiation system has temporal fidelity to in vivo myogenesis during murine embryogenesis. This feature allows the opportunity for the study of the cardiac developmental program in a time window where there is absence of interference from skeletal muscle.

The Mouse Embryonic Stem Cell Differentiation System as an In Vitro Model System for Cardiac Regional Specification—Cardiogenesis involves the expression of many genes that encode proteins required for muscle activity. Until recently, all the known mammalian cardiac chamber-specific protein isoforms were known to be co-expressed throughout the early looped heart, with establishment of regional specificity relatively late during cardiogenesis and, in some instances, following parturition (DeGroot et al., 1989; Lyons et al., 1990). Such is the case, for example, for the alkali myosin light chains, myosin heavy chains, and atrial natriuretic factor genes. However, recent evidence documenting early restriction of MLC-2V gene expression to the ventricular myocardium of the primitive heart tube prior to septation and the development of distinct cardiac chambers suggests that the molecular cues that lead to regional specification of some cardiac muscle cells may occur very early in development (O'Brien et al., 1993). This pattern of restricted expression of the cardiac MLC-2V gene provides a potential marker for examining patterning of the heart tube during the early stages of cardiogenesis.

During their differentiation in culture, ES cells express transcriptional and translational products of the MLC-2V gene, demonstrating that cardiac cell type specification occurs during in vitro cardiogenesis. Although the MLC-2V gene is expressed at high levels in both cardiac and slow skeletal muscles in mice, the demonstration that cardiac development precedes skeletal myogenesis in vitro supports the view that MLC-2V expression in beating bodies without detectable myogenin or MyoD mRNA is most likely derived from developing cardiac muscle. The co-expression of MLC-2V and ANF genes in individual cells following the dispersion of early stage differentiated aggregates, in addition to recent electrophysiological data documenting the expression of tetrodotoxin-resistant Na* channels, provide further compelling evidence for the cardiac-like nature of these cells.

The demonstration of the expression of a ventricular-specific muscle marker in an in vitro system that lacks a primordial heart tube, in the present study, provides evidence that regional specification can occur independently of positional cues or physiologic stimuli associated with the development of functional cardiac chambers. This finding suggests that the mechanisms that restrict gene expression to specific muscle cells may not be totally dependent upon formation of a heart tube and the associated hemodynamic influences. The question remains as to whether specification of the MLC-2V gene during the earliest stages of cardiogenesis results from the direct commitment of mesodermally derived precursor cells to the ventricular muscle lineage without an intermediary cardiac muscle stem cell, or whether a common cardiac muscle progenitor cell exists and gives rise to both atrial and ventricular myocytes.

The analysis of single cells derived from pools of beating embryoid bodies demonstrates at least four subpopulations of cells based on MLC-2V and ANF gene expression. We have recently developed techniques for the harvesting and purification of cardiac muscle cells from beating, differentiated embryoid bodies based on modification of previous protocols for the isolation and culture of primary cardiomyocytes. These cells also display the same immunofluorescence phenotypes, thereby confirming these findings. Under these conditions, the various cell types are detected at the following frequency: MLC-2V-positive/ANF-positive, 7%; MLC-2V-positive/ANF-negative, 44%; MLC-2V-negative/ANF-positive, 13%; and MLC-2V-negative/ANF-negative, 36%. These results further establish the mouse ES cell system as a model for cardiac chamber specification and suggest the possibility of tracking effects on these individual cell populations following genetic alterations of the parental ES cell lines.

Conclusions—In summary, our findings demonstrate that the embryonic stem cell system can model certain aspects of early cardiac muscle commitment and differentiation. The finding of a ventricular-specific marker in differentiated ES cells suggests that this may serve as a promising approach in the elucidation of the regulatory mechanisms that determine cell fate and control the expression of specific genes in specialized cell types during cardiogenesis. Modification of specific candidate genes via homologous recombination in ES cells should allow the opportunity to explore potential influences of targeted genes on the cardiac muscle program and cardiac chamber specification in vitro. This system should also provide a valuable approach for exploring genetic manipulations within an in vitro cardiac context that may otherwise result in embryonic lethality in transgenic animals. The in vitro differentiation of mouse embryonic stem cells into cardiac muscle cells with atrial- or ventricular-specific properties may ultimately allow for studies of chamber specification in genetically engineered cardiac muscle cells, thus providing a useful tool in the dissection of developmental cardiogenesis. In addition to the powerful features described above, this system may provide for the generation of large amounts of embryonic material and the biochemical purification of factors with potential influences on cardiac growth and differentiation. Ultimately, this system may also allow the opportunity for the identification and isolation of cardiac cell progenitors.

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3 M. E. Buckingham, personal communication.
4 T. Bahnson, S. J. Fuller, W. C. Miller-Hance, and K. R. Chien, unpublished observations.

5 S. J. Fuller, W. C. Miller-Hance, M. LaCorbiere, and K. R. Chien, manuscript in preparation.
In Vitro Model for Ventricular Specification

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REFERENCES

Adolf, E. A., Subramaniam, A., Ceereji, P., Olson, E. N., and Robbins, J. (1993) J. Biol. Chem. 268, 5549-5552
Barton, P. J. R., Robert, B., Cohen, A., Garner, L., Sassoon, D., Weydert, A., and Buckingham, M. (1986) J. Biol. Chem. 263, 12569-12576
Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990) Cell 61, 49-59
Chien, K. R. (1993) Science 260, 916-917
Chien, K. R., Knowlton, K. U., Zhu, H., and Chien, S. (1992) FASEB J. 6, 3037-3046
Chien, K. R., Zhu, H., Knowlton, K. U., Miller-Hance, W., van Bilsen, M., O'Brien, T. X., and Evans, S. M. (1993) Annu. Rev. Physiol. 55, 77-95
DeGroot, I. M., Lamers, W. H., Los, J. A., and Moorman, A. F. M. (1989) Anat. Rec. 224, 365-373
Doetschman, T. C., Ristetter, H., Katz, M., Schmidt, W., and Kemler, R. (1985) J. Embryol. Exp. Morphol. 87, 27-45
Evans, M. J., and Kaufman, M. H. (1981) Nature 292, 154-156
Farrance, I. K. G., Mar, J. H., and Ordahl, C. P. (1992) J. Biol. Chem. 267, 17234-17240
Iwaki, K., Sukhatsme, V. P., Shubeita, H. E., and Chien, K. R. (1990) J. Biol. Chem. 265, 13639-13647
Keller, G., Kennedy, M., Papayannopoulou, T., and Wiles, M. V. (1993) Mol. Cell. Biol. 13, 473-486
Knowlton, K. U., Baracchini, E., Ross, R. S., Harris, A. N., Henderson, S. A., Evans, S. M., Glembocki, C. C., and Chien, K. R. (1991) J. Biol. Chem. 266, 7759-7768
Lee, K. J., Ross, R. S., Rockman, H. A., Harris, A. N., O'Brien, T. X., van Bilsen, M., Shubeita, H. E., Randolf, K., Brem, G., Price, J., Evans, S. M., Zhu, H., Franz, W.-M., and Chien, K. R. (1993) J. Biol. Chem. 268, 15875-15885
Lindenbaum, M. H., and Grosveld, F. (1990) Genes & Dev. 4, 2075-2085
Lyons, G. E., Schiaffino, S., Sassoon, D., Barton, P., and Buckingham, M. (1990) J. Cell Biol. 111, 2427-2436
Mahdavi, V., Periasamy, M., and Nadal-Ginard, B. (1982) Nature 297, 659-664
Martin, G. R. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 7634-7638
Metcalf, D. (1991) Int. J. Cell Cloning 9, 95-108
Molkenstein, J. D., Bregan, R. S., Jabe, S. M., and Markham, B. E. (1993) J. Biol. Chem. 268, 2602-2609
Muthuchamy, M., Pajak, L., Howies, P., Doetschman, T., and Wieczorek, D. F. (1990) Mol. Cell. Biol. 10, 3231-3239
Navankasertussis, S., Zhu, H., Garcia, A. V., Evans, S. M., and Chien, K. R. (1993) Mol. Cell. Biol. 13, 1469-1479
O'Brien, T. X., Lee, K. J., and Chien, K. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5157-5161
Olson, E. N. (1993) Circ. Res. 72, 1-6
Pollock, R., and Treisman, R. (1991) Genes & Dev. 5, 2327-2341
Russo, W., Sarola, H. G., Sasse, E., Eklom, P., Kemler, R., and Doetschman, T. (1988) Development 102, 471-478
Robbins, J., Guldick, J., Sanchez, A, Howies, P., and Doetschman, T. (1990) J. Biol. Chem. 265, 11905-11909
Robbins, J., Doetschman, T., Jones, W. K., and Sanchez, A. (1992) Trends Cardiovasc. Med. 2, 44-49
Sanchez, A., Jones, W. K., Guldick, J., Doetschman, T., and Robbins, J. (1991) J. Biol. Chem. 266, 22419-22426
Schmitt, R. M., Broyne, E., and Snodgrass, H. R. (1991) Genes & Dev. 5, 728-746
Shubeita, H. E., McDonough, P. M., Harris, A. N., Knowlton, K U., Glembocki, C. C., Brown, J. H., and Chien, K. R. (1990) J. Biol. Chem. 265, 20555-20562
Thompson, W. R., Nadal-Ginard, B., and Mahdavi, V. (1991) J. Biol. Chem. 266, 25275-25285
Wang, R, Clark, R., and Bautch, V. L. (1992) Dev. Growth 114, 303-316
Weintraub, H., Davis, R., Tappson, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., Holleman, S., Zhuang, Y., and Lasas, A. (1991) Science 251, 781-786
Weidert, A., Daubas, P., Lacomb, I., Barton, P., Garner, I., Leader, D. P., Bonhommme, P., Catalon, D., Simon, D., Gfies, J. L., Gós, P., and Buckingham, M. E. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7183-7187
Wieczorek, D. F., Howies, P., and Doetschman, T. (1990) in The Dynamic State of Muscle Fibers (Pette, D., ed.) pp. 91-101, Walter de Gruyter, New York
Yu, Y.-F., Braebhert, R. E., Smoot, I. B., Lee, Y., Mahdavi, V., and Nadal-Ginard, B. (1992) Genes & Dev. 6, 1783-1798
Zhu, H., Garcia, A. V., Ross, R. S., Evans, S. M., and Chien, K. R. (1991) Mol. Cell. Biol. 11, 2273-2281
Zhou, H., Nguyen, V. T. B., Brown, A. B., Poussoneville, A., Garcia, A. V., van Bilsen, M., and Chien, K. R. (1993) Mol. Cell. Biol. 13, 4432-4444