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# Gradients of transgene expression directed by the human myoglobin promoter in the developing mouse heart

(gene regulation/cardiac development)

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ABSTRACT **Prior studies using transient transfection assays in cultured avian and murine skeletal myotubes indicate that the proximal 2-kb segment of the 5′ flanking region of the human myoglobin gene contains transcriptional control elements sufficient to direct muscle-specific and developmentally regulated expression of reporter genes. To examine the function of the human myoglobin gene promoter during development of skeletal and cardiac myocytes in the intact animal, a 2.0-kb myoglobin gene upstream fragment was fused to an *Escherichia coli lacZ* reporter gene and injected into fertilized mouse oocytes. β-Galactosidase (β-gal) activity was detected selectively in cardiac and skeletal myocytes of fetal and adult transgenic mice. A distinctive spatial pattern of myoglobin promoter activity was observed in fetal hearts: β-gal staining was more pronounced within the left ventricular subendocardium than within the subepicardium and was essentially undetectable in the ventricular trabeculae or atria. Expression of endogenous myoglobin mRNA and protein, assessed by *in situ* hybridization and immunohistochemistry, demonstrated a similar spatial pattern. In contrast, hearts from adult transgenic mice demonstrated essentially homogeneous expression of β-gal and of endogenous myoglobin mRNA and protein throughout the myocardium, including the trabeculae and atria. These data indicate that the 2.0-kb upstream region of the human myoglobin gene includes cis-acting regulatory elements sufficient to direct transgene expression during murine cardiac development that is myocyte-specific and responsive to positional cues in a similar manner to the endogenous myoglobin gene.**

The discovery of the family of myogenic determination genes and the characterization of their protein products as components of heterooligomeric DNA-binding transcription factors have illuminated important features of the genetic control mechanisms that direct the development of skeletal myocytes (1–5). However, much less progress has been made in elucidating cis-acting transcriptional control elements and the cognate binding factors that mediate the development of the related but distinct cell lineage that forms cardiomyocytes. Moreover, many of the important phenotypic properties of both skeletal and cardiac myocytes are not determined exclusively by developmental programs but are regulated by physiological and metabolic stimuli that match the biochemical and structural characteristics of myocytes to the demand for contractile work (6–11).

Myoglobin is a cytosolic hemoprotein that is expressed selectively in cardiac and skeletal myocytes, where it functions to facilitate diffusion of oxygen and to maintain mitochondrial respiration during heavy and sustained contractile work and in the rest–work transition (12). Within fully differentiated myocytes, expression of myoglobin is exquisitely sensitive to changes in tonic contractile activity that increase the demand for ATP production via oxidative metabolism (13, 14). We previously have explored the developmental regulation of the myoglobin gene in skeletal myotubes and have identified a muscle-specific enhancer element within the 5′ flanking region of the human myoglobin gene (15–17). The present study was undertaken as a step toward elucidation of transcriptional control mechanisms that regulate the expression of myoglobin during cardiac development.

## MATERIALS AND METHODS

**Plasmid Construction.** A 2-kb segment of the 5′ flanking region of the human myoglobin gene extending from position −2038 to +7 relative to the cap site was removed as a *Pvu* II fragment from a genomic clone originally isolated by Weller *et al.* (18). This segment was subcloned into the *Hin*cII site of pBSM13+ (15), removed as a *Kpn* I–*Pst* I fragment, and inserted into the polylinker of pBluescript II KS(+) (Stratagene). The 2-kb fragment was subsequently removed and inserted as an *Xba* I fragment in correct orientation upstream of the *Escherichia coli lacZ* gene in pKSlacZ (provided by Radek Skoda, Harvard University). Enzymatically active β-galactosidase (β-gal) was expressed in primary cultures of skeletal myotubes derived from chicken embryos after transfection of the resultant construct pMB-2038lacZ (data not shown). A 5.8-kb *Not* I–*Kpn* I fragment including the myoglobin gene promoter, *lacZ* gene, and simian virus 40 splice and polyadenylylation signal [base pairs 2638–3507 from plasmid CDM8, provided by Brian Seed (Harvard University)] was excised, separated from plasmid sequences on a 1% agarose gel, and electroeluted prior to resuspension in sterile 1 mM Tris/0.1 mM EDTA, pH 7.4, at a final concentration of ≈3 ng/μl for microinjection.

**Transgenic Animals.** Microinjection of the transgene construct into fertilized mouse oocytes of strain FVB/N (Harlan–Sprague–Dawley) was performed by standard techniques (19). Fetal mice (18.5 days of gestation) and adult mice (28 days postnatal) were screened for the presence of the transgene by Southern blot hybridization after digestion of genomic DNA with *Spe* I (20). *Spe* I cuts once within the transgene, thereby providing an assessment of integration site and copy number. The probe was an 869-bp fragment containing simian virus 40 sequences identical to those of the transgene and was labeled by the random priming method (Boehringer Mannheim).

**Analysis of β-Gal Expression.** Dissected fetal tissues were fixed by immersion in 0.1 M Pipes/2% paraformaldehyde, pH 6.9, for 30 min. Tissues were washed three times in 0.1 M

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Abbreviation: β-gal, β-galactosidase.

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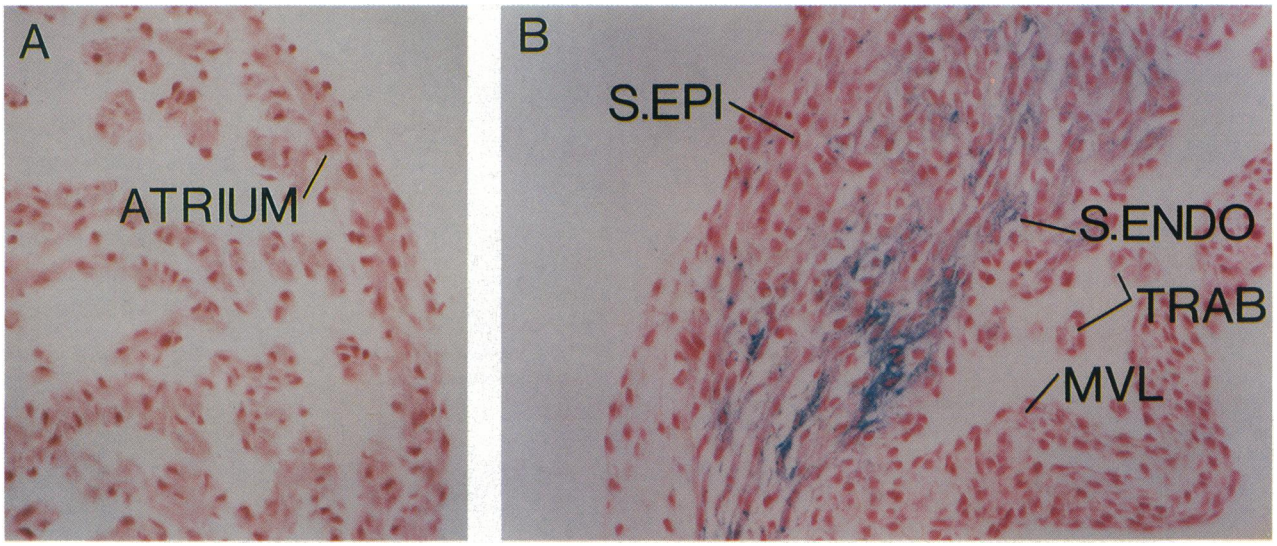


FIG. 1. Histological detection of β-gal expression in cardiac tissue of day 18.5 transgenic mouse embryo. Expression is undetectable in atrium (*A*) and mitral valve leaflet (MVL) (*B*) and is low in the ventricular trabeculae (Trab) (*B*). Expression is more prominent in subendocardial (S. Endo) than subepicardial (S. Epi) regions of the left ventricle (*B*). A similar pattern was observed in each of two transgenic animals representing independent lines. (×160.)

Pipes (pH 7.4) for 15 min at room temperature and stained overnight with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (21). Tissues were dehydrated in ethanol and cleared with xylene and cedar wood oil. For microscopic analysis of transgene expression, tissues were embedded in paraffin, sectioned at a thickness of 3 μm, and counterstained with nuclear fast red.

***In Situ* Hybridization.** A 503-bp fragment (exon 3) of the murine myoglobin gene was prepared by use of the polymerase chain reaction. This fragment was subcloned into the *Eco*RI/*Xba* I sites of pGEM-3Z (Promega), and its identity was confirmed by DNA sequencing (22). Radiolabeled antisense and sense probes were generated by *in vitro* transcription of linearized DNA templates using either phage T7 or SP6 RNA polymerase (Promega) in the presence of $^{35}$S-labeled UTP (1200 Ci/mmol, DuPont; 1 Ci = 37 GBq). Fetal and adult mouse hearts of strain FVB/N were fixed overnight at 4°C in 0.1 M phosphate-buffered saline/4% paraformaldehyde, pH 7.5. Tissues were dehydrated in ethanol, cleared with xylene, embedded in paraffin, sectioned at a thickness of 3 μm, and mounted on aminoalkylsilane-treated slides. Hybridizations to radiolabeled probes were performed as described by Wilkinson *et al.* (23) with modifications described by Frohman *et al.* (24).

**Immunohistochemical Analysis.** Tissue sections were prepared as described for *in situ* hybridization, deparaffinized, and incubated with rabbit anti-chicken myosin IgG antibody (gift of Woodring E. Wright, University of Texas Southwestern Medical Center, Dallas) or with goat anti-human myoglobin IgG antibody (Organon Teknika–Cappel). Both antibodies cross-react with their corresponding murine proteins (25). Primary antibodies were detected by using an immunoperoxidase procedure (Vectastain ABC kit; Vector Laboratories). Sections were counterstained with Harris hematoxylin.

## RESULTS

Southern blot hybridization analysis (not shown) revealed that, among the 12 offspring tested at a gestational age of 18.5

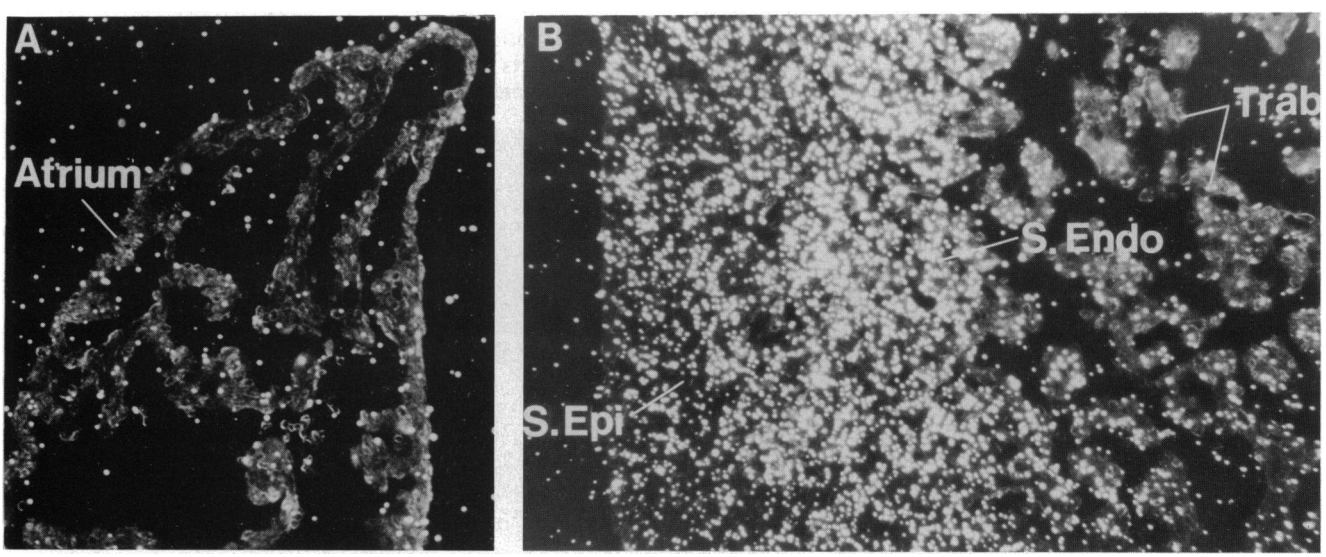


FIG. 2. Endogenous myoglobin mRNA in cardiac tissue of day 18.5 mouse embryo. Expression is essentially undetectable in atrium (*A*) and low in ventricular trabeculae (Trab) (*B*). Expression is more prominent in subendocardial (S. Endo) than subepicardial (S. Epi) regions of the left ventricle (*B*). A similar spatial pattern was observed in each of three fetal hearts. (×130.)

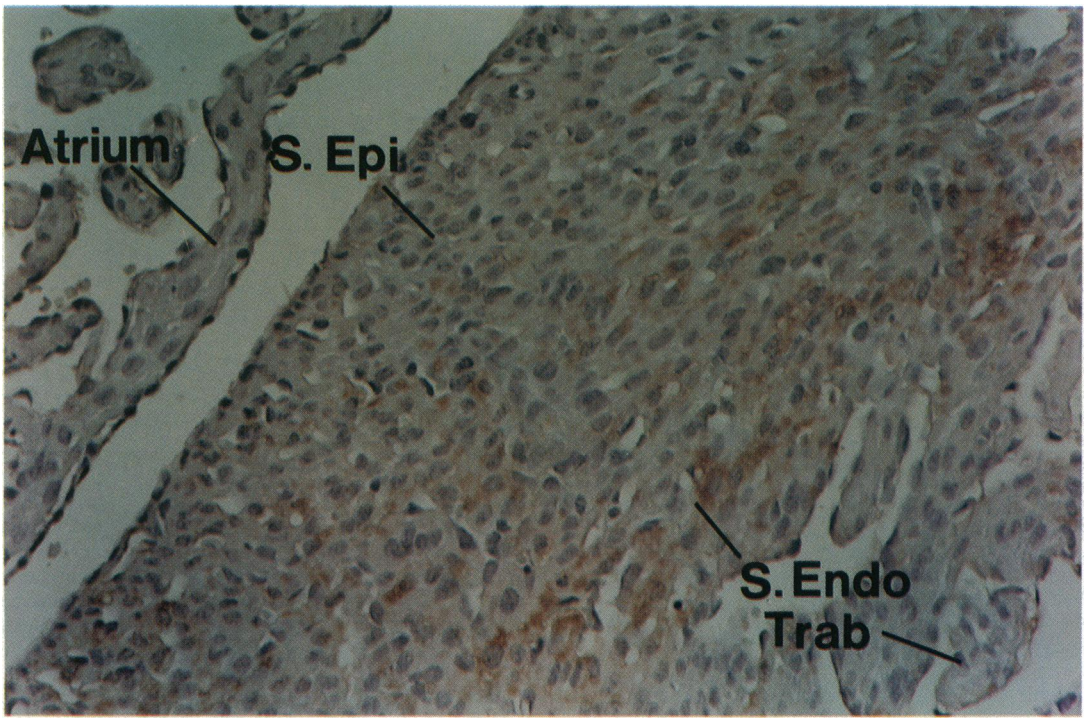


FIG. 3. Immunohistochemical detection of endogenous myoglobin in cardiac tissue of day 18.5 mouse embryo. Myoglobin protein is more abundant in subendocardial (S. Endo) than subepicardial (S. Epi) regions of the left ventricle and is essentially undetectable in atrium or ventricular trabeculae (Trab). A similar staining pattern was observed in each of three fetal hearts. (×160.)

days, integration of the transgene had occurred in 3 animals. Similarly, among 15 offspring tested at a postnatal age of 28 days, transgene integration had occurred in 4 animals. Variations in intensity and size of bands observed in the Southern blot reflected differences in copy number and orientation of the transgene inserts among these animals and indicated independent chromosomal integration events. Endogenous β-gal activity was evident in small intestine and kidney of all animals (not shown) irrespective of the presence of the transgene, as described previously (26). In 2 of the 3 fetal transgenic animals and in 2 of the 4 adult transgenic animals, β-gal activity was expressed within the hearts. In these animals transgene expression also was detected in skeletal muscle, but not in brain, liver, lung, or spleen.

Microscopic analysis of β-gal expression in the fetal heart is shown in Fig. 1. A similar pattern of staining was observed in both transgene-positive fetal mice that expressed β-gal activity. Transgene expression was limited to cardiomyocytes and was absent in nonmyocytes within the ventricular wall and valves. Of note, β-gal activity within the left ventricular wall was more prominent in the subendocardium than in the subepicardial regions. In addition, the ventricular trabeculae and atria were devoid of β-gal activity. Analysis of endogenous myoglobin mRNA and protein by *in situ* hybridization and immunohistochemistry, respectively, revealed a similar spatial pattern at this developmental stage (Figs. 2 and 3). In contrast, other muscle-specific genes, such as that encoding cytochrome oxidase subunit VIa (heart isogene), exhibited uniform mRNA expression in atria and ventricles and in the inner and outer regions of the left ventricular wall (data not shown). Likewise, at the protein level, an antimyosin antibody showed identical reactivity in all myocardial regions (data not shown).

The spatial gradients of myoglobin promoter activity in fetal hearts appear to be a transient feature of cardiac development, since the distribution of β-gal activity in hearts

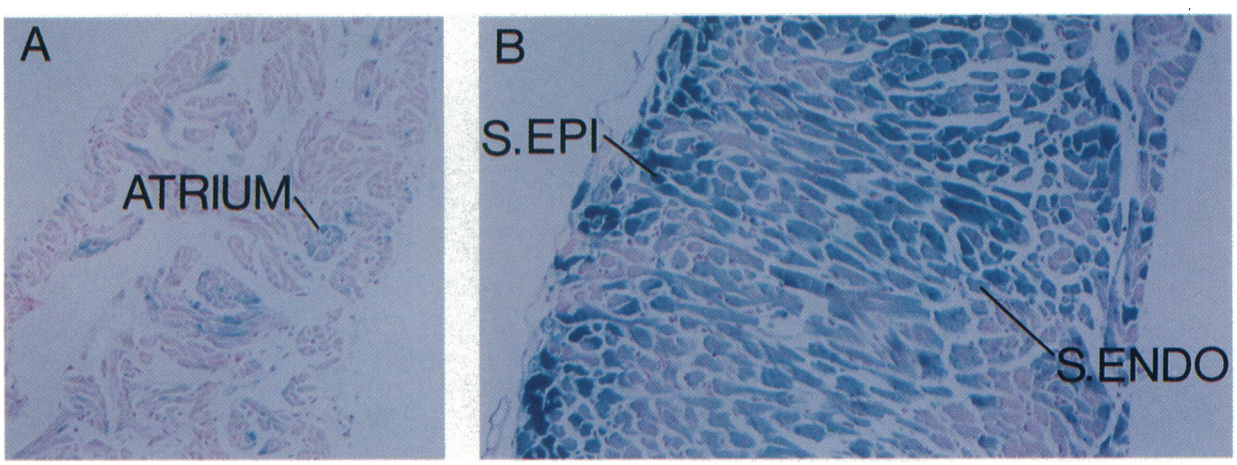


FIG. 4. Histological detection of β-gal expression in cardiac tissue of adult transgenic mouse. Transgene expression is present in atrium (*A*) and is similar in subepicardial (S. Epi) and subendocardial (S. Endo) regions of the left ventricle (*B*). A similar pattern was observed in each of two transgenic animals representing independent lines. (×80.)

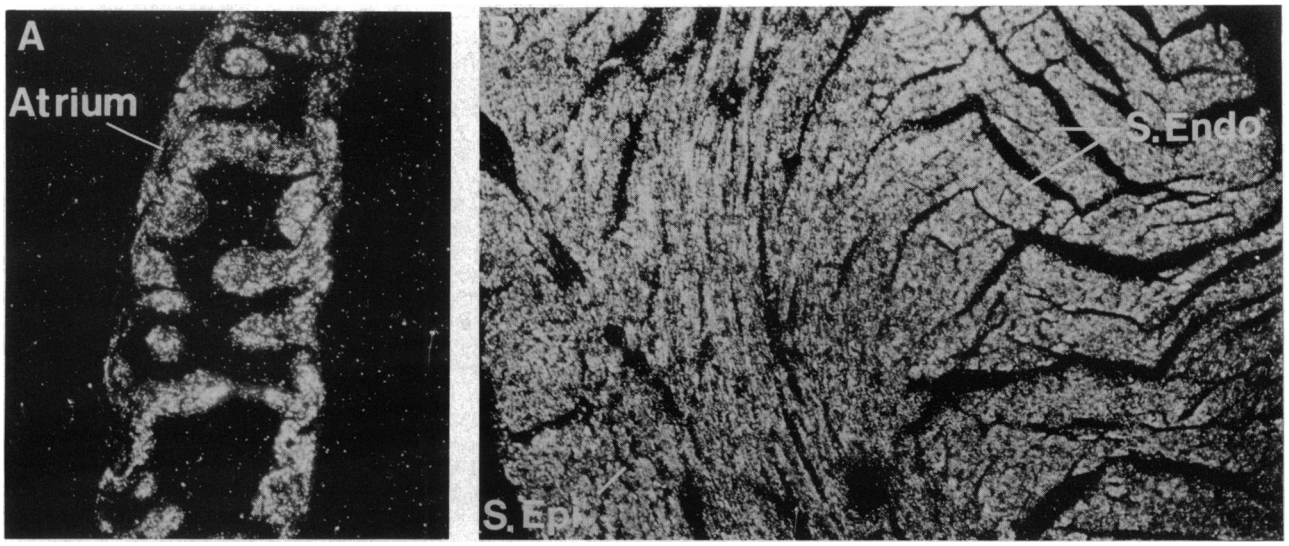


FIG. 5. Expression of endogenous myoglobin mRNA in cardiac tissue of adult mouse. Expression is present in atrium (*A*) and is similar in subepicardial (S. Epi) and subendocardial (S. Endo) regions of the left ventricle (*B*). This expression pattern was observed in each of three adult hearts. (×65.) Control experiments using a sense probe revealed only a uniform pattern of low-level nonspecific hybridization (not shown).

of adult transgenic mice was similar in subepicardial and subendocardial regions of the left ventricle (Fig. 4). In addition, β-gal activity was readily detected in the atria and trabeculae. Expression of endogenous myoglobin mRNA and protein likewise was similar within all regions of the adult mouse heart (Figs. 5 and 6, respectively).

## DISCUSSION

A principal finding of this study is that the immediate upstream region (2 kb) of the human myoglobin gene includes cis-acting regulatory elements sufficient to localize expression of a heterologous reporter gene to cardiac and skeletal myocytes during prenatal development of the mouse. A second major finding is that the 2.0-kb fragment also includes regulatory elements sufficient to confer spatially graded expression of the transgene in fetal hearts in a manner similar to the endogenous myoglobin gene.

With respect to skeletal muscle development, these results confirm the conclusions drawn from transient transfection assays of primary cultures of skeletal myotubes derived from chicken embryos (15). This region of the human myoglobin gene contains proximal promoter elements (TATA) and several sequence motifs shared among other genes that are expressed in a muscle-specific manner. Two E box motifs that are likely to comprise binding sites for the members of the basic helix–loop–helix family of myogenic determination genes (27), a putative myocyte-enhancing factor 2 motif (28, 29), and a CCAC motif (30) are present within the 2.0-kb upstream region of the human myoglobin gene (15–17). We have employed fine-structure mutational analysis of these elements in transient transfection assays of cultured myotubes to define the relative contributions of these elements to transcriptional activation during skeletal muscle differentiation (15–17). The findings in the current study indicate the feasibility of extending this type of detailed analysis to both skeletal and cardiac muscle in the developing mouse embryo.

The cis-acting elements that mediate transcriptional activation of the myoglobin gene during cardiac development have not been mapped. The fact that such elements are

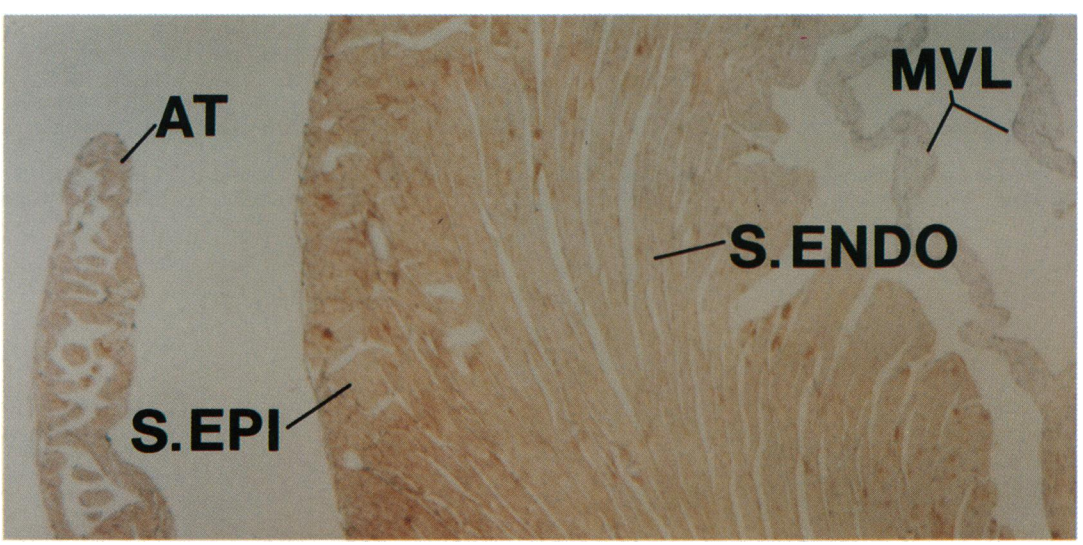


FIG. 6. Immunohistochemical detection of endogenous myoglobin in cardiac tissue of adult mouse. Myoglobin protein is present in atrium, is similar in subepicardial (S. Epi) and subendocardial (S. Endo) regions of the left ventricle, and is absent in the mitral valve leaflet (MVL). This staining pattern was observed in each of three adult hearts. (×30.)

included within the proximal 2 kb of 5′ flanking DNA provides the basis for further experiments to localize these sequences at higher resolution. Definition of transcriptional control elements that have this function may facilitate characterization of the cognate transcription factors within cardiomyocytes that have roles analogous to those served by myogenic determination genes in skeletal muscle differentiation (1–5).

Although a number of endogenous genes manifest a changing spatial pattern of expression during early embryogenesis of cardiac and skeletal muscle (31, 32), the regional heterogeneity in activity of the myoglobin promoter that we observed in the heart late in murine gestation was unanticipated. It is unlikely that this pattern is due to inhomogeneity of fixation because β-gal activity in the thicker walls of adult ventricles was essentially uniform in subepicardial and subendocardial regions. Donoghue *et al.* (33) and Grieshammer *et al.* (32) recently described a rostrocaudal gradient of transgene expression in certain axial skeletal muscles of mice in which a chloramphenicol acetyltransferase (CAT) reporter gene was controlled by a rodent myosin light-chain promoter. Our results suggest that positional regulators play an important role in specifying gradients of gene expression during cardiac as well as skeletal muscle development and that this role is not limited to early stages of embryogenesis. Our data also indicate that specific transcriptional control elements within the 2.0-kb upstream region of the human myoglobin gene are responsive to regional differences in intracellular signals in a manner analogous to the endogenous gene. The use of transgenic models to analyze further the transcriptional control elements contained within the myoglobin promoter may help to elucidate pathways by which positional information is transduced during development of the mammalian heart.

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