Growth and Partial Differentiation of Presumptive Human Cardiac Myoblasts in Culture

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Abstract. A cell culture model for human cardiac myogenesis is introduced. Human fetal myocardial cells were dissociated enzymatically, and cultured in a mitogen-rich medium that promoted the growth of presumptive cardiac myoblasts. Strains of human cardiac myoblasts were generated from different anatomical regions of the fetal heart. The cells could be cultured for at least 30 generations, or frozen and recovered for later use. Differentiation was induced by culturing the cardiac myoblasts in a mitogen-poor medium. Differentiation of cardiac myoblasts was marked primarily by transcriptional activation of the atrial natriuretic factor (ANF) gene. Evidence is presented that posttranscriptional processing of ANF transcripts is affected by the anatomical origin of the cardiac myoblasts and the presence of cocultured neuronal cells. Cardiac myoblasts induced to differentiate in culture synthesized only low levels of sarcomeric myosin and cardiac α-actin, suggesting that differentiation of these cells progresses through two phases: an initial, noncontractile phase that is represented by the differentiating cultured cells; and a later contractile phase, in which myofibrillar assembly is accentuated and modulated by secondary signals from the cardiac milieu.

STUDIES of cell differentiation are a focal point for understanding the process of organ and tissue development. One effective way to study cell differentiation is by culturing committed progenitor cells (sometimes derived from tumor cells), and, through alterations in the culture conditions, inducing them to differentiate. Although the development of several types of tissue has been represented by such culture systems, others are unrepresented. A tissue culture model for myocardial development amenable to continuous passaging has not been described. When compared to the elegant culture systems available for studying the development of skeletal muscle, the absence of a culture system for studying the development of cardiac muscle is surprising.

Distinct stages in the development of fetal, perinatal, and adult cardiac myocytes can be recognized by morphological (Manasek, 1970; Sreter et al., 1975), biochemical or immunochemical (Hoh et al., 1978; Sartore et al., 1978; Flink et al., 1978; Price et al., 1980; Lompre et al., 1981; Samuel et al., 1983; Lambert et al., 1983; Cummins and Lambert, 1986; Claycomb, 1986), and recently identified molecular (Mahdavi et al., 1982, 1984; Lompre et al., 1984; Izumo et al., 1985; Nadal-Ginard et al., 1987) criteria. In addition, expression of the major secretory product of the heart, atrial natriuretic factor (ANF;[1] reviewed by deBold, 1985), appears to be modulated during myocardial development (Bloch et al., 1986). The conditions influencing the maturation of cardiac myocytes and the modulation of their contractile and secretory phenotypes, however, are not well understood. Although cardiac myocytes can be dissociated from the myocardium and maintained in culture (Powell and Twist, 1976), these cells do not proliferate appreciably and thus represent an incomplete developmental model. The generation of a culture system for progenitor cells of cardiac myocytes (cardiac myoblasts) would allow the proliferation and subsequent differentiation of myocardial cells, and would serve as a useful in vitro model of cardiac gene expression during development.

Important differences have been observed between the biochemical events that occur during cardiac development in different species (Yazaki and Raben, 1974; Lompre et al., 1981; Clark et al., 1982; Syrovy, 1982; Cummins and Lambert, 1986), emphasizing the need to document studies of cardiac development in a human culture system. The proliferation and differentiation of human cardiac myoblasts in culture are presented here as a model for human cardiac gene expression during development. Human fetal cardiac myocytes were enzymatically dissociated from autopsy material,

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1. *Abbreviations used in this paper*: ANF, atrial natriuretic factor; FGF, fibroblast growth factor; MSA, multiplication-stimulating activity factor.

and cultured in mitogen-rich medium containing basic fibroblast growth factor (FGF; Gospodarowicz, 1975) and multiplication-stimulating activity factor (MSA; Dulak and Temin, 1973). Some of the cells proliferated in this medium and within a few passages the cultures did not express sarcomeric isoforms of myosin heavy chain. Cultures of the replicating cells (cardiac myoblasts) could be maintained for at least 30 generations, and frozen stocks were generated. Strains of human cardiac myoblasts were derived from right atrium (A1), total ventricle (HAM-1), left ventricle (LV-1), and right ventricle (RV-1) of 20–22-wk fetal autopsy material. When switched to mitogen-poor medium, these cells initiated a program of differentiation, expressing low levels of sarcomeric muscle myosin and cardiac α-actin. The most prominent marker of differentiation observed in these cells was the induction of ANF mRNA expression. Posttranscriptional regulation of ANF mRNA expression by differentiating human cardiac myoblasts was also observed. The low levels of sarcomeric mRNAs and proteins expressed by the differentiated cardiac myoblasts suggests that the phenotype of these cells represents an intermediate in the development of fully functional cardiac myocytes, and that additional cues are necessary to induce their maturation.

Materials and Methods

Materials, Probes, and Cell Lines

SH-SY5Y cells (given by S. Berl, Department of Neurology, Mount Sinai School of Medicine, New York; Perez-Polo et al., 1979) were adapted to growth in 4% horse serum. The following plasmid clones were used as probes in this study: phH5B (histone H3; Heintz et al., 1983), pHMcA-3′UT-DB (cardiac α-actin; Gunning et al., 1983; Bains et al., 1984), pGH33 (preproANP; Maki et al., 1984), and pI-19 (murine 28-S ribosomal RNA; Tiemeirer et al., 1977; given by the laboratory of S. Chen-Kiang, Mount Sinai School of Medicine). Ascites fluid for monoclonal antibody MF-20 (Bader et al., 1982) was generated in pristane-primed nude mice (J. Kohtz, Department of Pathology, Mount Sinai School of Medicine).

Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT), tissue culture reagents were purchased from Gibco Laboratories (Grand Island, NY), and plasticware was purchased from Corning Glass Works (Corning, NY). Chick embryo extract (50%) was prepared from 7–9-d chick embryos. Embryos were mixed with an equal volume of normal saline, and homogenized in a Waring Products (New Hartford, CT) blender. The homogenates were clarified by centrifugation at 30,000 *g*, and sterilized by filtration (0.22 μm cellulose-acetate filter).

Culturing Human Cardiac and Skeletal Myoblasts

Samples of fetal heart tissue were aseptically obtained from autopsy specimens (voluntary and spontaneous abortions). After dissection, the tissue samples were suspended in Hank's Balanced Salts Solution minus calcium and magnesium (HBSS; Gibco Laboratories) containing penicillin (50 U/ml), streptomycin (50 U/ml), and gentamycin (100 μg/ml). Samples were washed twice with this solution, minced with sterile scissors into 2–3-mm-diam fragments, and placed in 250-ml, single-neck, digestion flasks. The samples were then digested with stirring for 30 min (37°C) in HBSS containing 1 mg/ml collagenase (type III) and 50 μM CaCl. The released material was decanted, and the samples were digested for 30 min (37°C) with a fresh solution of HBSS containing 300 μg/ml elastase. The released material was decanted, and the collagenase digestion was repeated. Cells released by the predigestions were used for fibroblast cultures. After the released material was decanted, the remaining material was digested with 25 μg/ml trypsin in HBSS for 30 min (37°C). The trypsin digestion was repeated three times, and the released cells were recovered from each digestion. The solutions containing the released cells were made 5% in fetal bovine serum (to inactivate the trypsin) and pooled. The cells were pelleted and washed three times in HBSS (600 *g* for 10 min; then twice at 300 *g* for 10 min).

The trypsin-dissociated cells were plated in mitogen-rich medium (DME supplemented with 20% fetal bovine serum, 1.0% chick embryo extract, 50 ng/ml FGF [Collaborative Research, Inc., Waltham, MA], 25 ng/ml MSA [Collaborative Research, Inc.], 1 mM nonessential medium amino acids [Gibco Laboratories], 0.1 mM sodium pyruvate, 0.1 mM minimum essential medium vitamins [Gibco Laboratories]). The cells were plated at 10^4–10^5 adhering cells per 75-cm^2 culture flask, and routinely passed to 5×10^4 cells per flask. The cells were grown to confluence (1–2 wk; medium is changed every 3 d). The monolayers were dissociated with trypsin for passaging. Viable recovery of the cells was accomplished if they were frozen in 40% fetal bovine serum, 10% dimethyl sulfoxide, 40% RPMI (Gibco Laboratories). Strains of cardiac myoblasts could be derived using 1% chick embryo extract in the absence of exogenously added FGF and MSA. However, these strains grew slower, and appeared to eventually be overgrown with cells bearing the morphology of fibroblasts (Fig. 1 *c*).

Differentiation of the cells at confluence was induced by culturing in mitogen-poor medium (DME supplemented with 4% horse serum, 1 mM nonessential amino acids, 0.1 mM sodium pyruvate, and 0.1 mM minimum essential medium vitamins). Substituting insulin (1 μg/ml), transferrin (1 μg/ml), and sodium selenite (1 ng/ml) for horse serum in this medium did not result in the induction of ANF expression in cardiac myoblasts, although cardiac α-actin expression was still observed (not shown).

Human cardiac myoblasts were separated from cocultured neuroblastoma cells by polycarbonate membranes (low protein adherence) containing either 0.45- (Millipore Corp., Bedford, MA) or 3.0- (Costar, Cambridge, MA) μm pores. Neuroblastoma cells (SH-SY5Y) were precultured on the membranes until they reached 50% confluence. Cardiac myoblasts were precultured on petri-style plates in growth medium to 90% confluence, then switched to differentiation medium. The SH-SY5Y cells were then overlaid on the cardiac myoblasts in such a manner that the neuroblastoma cells were on one side of the membrane and the cardiac myoblasts were on the other. Since the cardiac myoblasts had adhered to the culture plates, the SH-SY5Y cells could be easily separated from them later by removing the membrane overlay. Rims on the filters extending above the level of the culture medium prevented migration of SH-SY5Y cells into the culture plates containing the cardiac myoblasts.

A sample of thigh muscle from a 22-wk aborted fetus was processed and cultured identically to the heart tissue (above), resulting in the generation of the HUSK-1 strain of skeletal myoblasts. HUSK-1 skeletal myoblasts could be induced to differentiate into myotubes in mitogen-poor medium (described above) or in mitogen-poor medium with insulin (1 μg/ml), transferrin (1 μg/ml), and sodium selenite (1 ng/ml) substituted for horse serum.

Isolation of Myofibrillar Proteins from Differentiated HAM-1 Cells

Myofibrillar proteins were isolated by a modification of a protocol described elsewhere (Etlinger et al., 1976). Cardiac myoblasts were scraped from their culture dishes, and homogenized with a Dounce homogenizer (Kontes Glass Co., Vineland, NJ; B pestle) in PB buffer (0.1 M KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreital, 2 mM $Na_4P_2O_5$, 10 mM Tris maleate, pH 6.8). The homogenate was spun at 800 *g* for 10 min, and the pellet was then washed four times with PB buffer, and four times with PB buffer minus pyrophosphate. The final pellet was washed once with PB buffer minus pyrophosphate, supplemented with 0.1% Triton X-100. The preparations could be stored in 50% glycerol/PB buffer minus pyrophosphate.

SDS-PAGE Western blotting, and Coomassie Blue stains were performed as reported elsewhere (Kohtz et al., 1987) without substantial modifications.

Nuclear Run-on Transcription Assays

The protocols used for nuclear run-on transcript assays were modified from those described by Greenberg and Ziff (1984). Nuclei were prepared from cells at various phases of their differentiation program. One 15-cm-diam petri-style plate or two 75-cm^2 culture flasks were used per experimental data point. Cell monolayers were washed twice with warmed normal saline, then treated directly with 2 ml of NP-40 extraction buffer (10 mM Tris, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, and 1 mM dithiothreital). The cells were incubated on ice for 5 min in this buffer, then scraped off the dishes with a rubber policeman. The resulting suspension was vortexed three times, then pelleted at 500 *g*. The supernatant was removed, mixed with vanadyl ribonucleoside complexes, and used for the isolation of cytoplasmic RNA (below). The nuclear pellet was washed once with NP-40 extraction buffer, then flash frozen and stored (in liquid nitrogen) in 100 μl of 50 mM Tris, 40% glycerol, 5 mM $mgCl_2$, 0.1 mM EDTA, and 1 mM dithiothreital.

A 2× reaction buffer was added to the isolated nuclei to initiate run-on transcription: 10 mM Tris, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 1 mM dithiothreital, 500 μM ATP and CTP, 100 μM UTP and GTP, and 100 μCi each of α-^{32}P-labeled UTP and GTP. The reactions were allowed to proceed for 20 min at 31°C before being stopped with an equal volume of 100 mM EDTA, 2% SDS (pH 8.0). The suspensions were then extracted twice with phenol/chloroform (first 2:1, then 1:1). 1/10 vol of 3.0 M sodium acetate (pH 5.0) was added, and the run-on transcripts were precipitated with 2.5 vol of ethanol. The transcripts were further purified from free nucleotide by Sephadex G-50 column chromatography.

Plasmids were isolated from HB101 by a standard Triton X-100 lysis/cesium chloride density gradient centrifugation procedure (Maniatis et al., 1982). The binding of restriction endonuclease–linearized, heat-denatured probes to nitrocellulose filters was performed in a sodium acetate buffer as described by the manufacturer of the slot-blotting apparatus that was used (Schleicher and Schuell, Inc., Keene, NH). Equivalent disintegrations per minute of the isolated run-on transcripts were denatured briefly in water at 70°C, then suspended in a hybridization mixture (50% formamide, 2× SSC [1× SSC is 0.15 NaCl, 15 mM Trisodium citrate, pH 7.0], 10% dextran sulfate, 1% SDS, 10 mM Tris, pH 7.0, 1 mM EDTA, 0.5% BSA, 0.5% polyvinylpyrrolidine, 0.5% Ficoll [mol wt 400,000], 10 μg/ml poly A, 100 μg/ml denatured *Escherichia coli* DNA). Nitrocellulose filters were prehybridized with this mixture overnight at 42°C, then hybridized to the run-on transcripts for 30 h at 42°C. The filters were then washed twice with 0.5× SSC and 1% SDS at 56°C, then twice with 0.2× SSC at 42°C. The filters were autoradiographed with Eastman Kodak Co. (Rochester, NY) X-AR5 film using Dupont Co. (Wilmington, DE) Lightening plus intensifying screens.

Northern Blot Analysis

Total cytoplasmic RNA was isolated from the first supernatants of the nuclear isolation procedure (see above). Vanadyl ribonucleoside complexes were removed from these supernatants by centrifugation (20,000 *g* for 10 min). Clarified cytosols were then diluted 1:1 with 1% SDS, 100 mM EDTA, 40 mM Tris (pH 7.0), and 200 μg/ml proteinase K. The mixtures were incubated for 1 h at 37°C, then extracted three times with phenol/chloroform (2:1, then twice at 1:1). 1/10 vol 3.0 M sodium acetate (pH 5.0) was added, and the RNA was precipitated with 2.5 vol of ethanol.

The RNA was dissolved and denatured in a buffer containing formaldehyde and formamide, and resolved on a formaldehyde/agarose gel as described (Maniatis et al., 1982). The RNA was then transferred to a nylon filter (GeneScreen plus; New England Nuclear, Boston, MA) electrophoretically in 12 mM Tris, 6 mM sodium acetate, 0.3 mM EDTA, pH 7.5, and baked in vacuo for 2 h at 80°C to reverse the formaldehyde reaction. Linearized plasmid DNA was nick translated as described elsewhere (Rigby et al., 1977), and purified from free nucleotides by Sephadex G-50 column chromatography. Nylon filters were prehybridized overnight with 2% SDS, 2× SSC, 10% dextran sulfate, 100 μg/ml denatured *E. coli* DNA, 10 μg/ml poly A, 50% formamide at 42°C. They were then hybridized with 5×10^5 dpms/ml of probe in a new batch of the same mixture (42°C for 30 h). The filters were washed twice in 2× SSC containing 1% SDS (56°C), twice with 0.2× SSC with 1% SDS (56°C), and twice with 0.1× SSC (room temperature). Autoradiography was performed with Eastman Kodak Co. XAR-5 x-ray film and Dupont Co. Lightening plus intensifying screens.

Results

Generation of Human Cardiac Myoblast Cultures

Samples of fetal heart tissue (20–22 wk) were digested sequentially with collagenase, elastase, collagenase, and trypsin. The cells released during the trypsin digestions were recovered, and cultured on standard tissue culture–grade plasticware. Three types of cells could be observed in these cultures: fetal cardiac myocytes (Fig. 1 *a*), which were mature cells that expressed contractile proteins and were capable of rapid spontaneous contractions; cardiac fibroblasts (Fig. 1 *c*), which did not express contractile proteins and were distinguished by their flat appearance and large nuclei; and cardiac myoblasts (Fig. 1 *b*), which were smaller, more granular cells than cardiac fibroblasts. Culturing dissociated fetal heart cells in DME containing 10% fetal bovine serum did not promote the growth of cardiac myoblasts, so that the slowly proliferating cardiac fibroblasts eventually overgrew the cardiac myocytes in these cultures. Culturing dissociated fetal heart cells in mitogen-rich medium (DME, 20% fetal bovine serum, 1% chick embryo extract, 50 ng/ml FGF, and 25 ng/ml MSA) allowed the rapid proliferation of cardiac myoblasts, so that by the third passage these cells dominated the cultures. Individual strains of human cardiac myoblasts could be maintained for at least 30 generations, or frozen and recovered for specific experiments. Strains of these cells were prepared from total ventricle (HAM-1), left ventricle (LV-1), right ventricle (RV-1), and right atrium (A1).

Differentiation of human cardiac myoblasts was induced by changing the cultures from mitogen-rich to mitogen-poor medium (DME + 4% horse serum). Cessation of mitosis in this medium was evident after 2 d by a >90% reduction in [^{3}H]thymidine incorporation (not shown), and by a signifi-

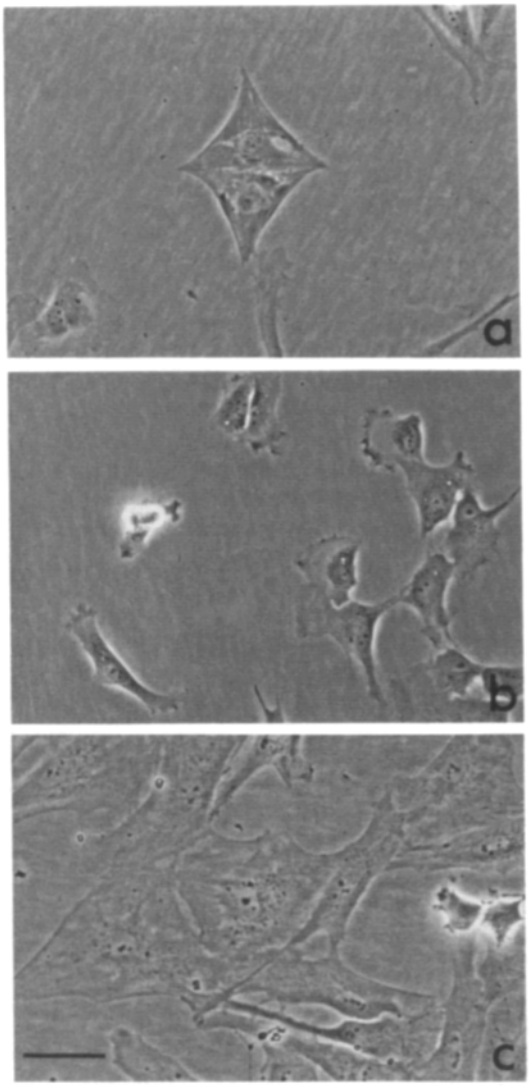


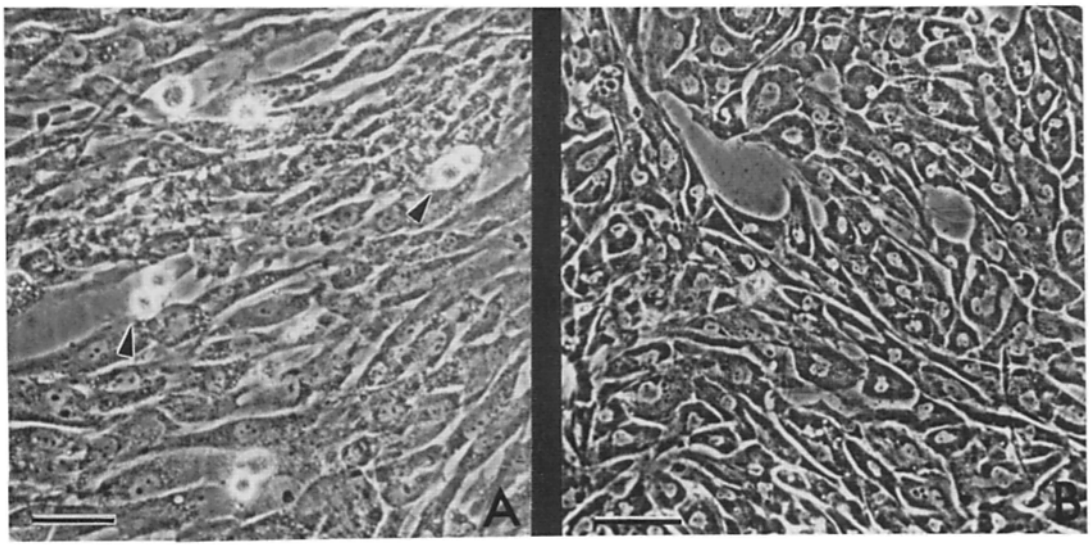
Figure 1. Phase-contrast microscopy of three types of cells released by enzymatic digestion of fetal heart tissue. (*a*) Fetal cardiac myocytes (atrial; 7 d in culture). (*b*) Cardiac myoblasts (passaged strain). (*c*) Cardiac fibroblasts (passaged strain). Bar, 50 μm.

cant reduction in histone H3 transcription (discussed below). By phase-contrast, light microscopy (Fig. 2), however, cardiac myoblasts cultured in mitogen-poor medium did not resemble cultured fetal cardiac myocytes. This initial observation suggested that the cardiac myoblasts are induced by mitogen-poor medium to differentiate into a form of cardiac myocyte less mature than the fetal cardiac myocyte. Further characterization of the phenotype of cardiac myoblasts induced to differentiate in mitogen-poor medium was performed by electron microscopy. Cardiac myoblasts cultured in mitogen-rich medium contained numerous Golgi complexes and mitochondria but no myofibrils (Fig. 2 *C*); cardiac myoblasts induced to differentiate in mitogen-poor medium contained less Golgi complexes, numerous lysosomes, and primitive myofibrillar structures (Fig. 2 *D*). The myofibrillar structures contained discernable Z bands but poorly defined A, H, and M bands. Preliminary evidence derived from antititin monoclonal antibodies suggests that these structures may contain titin (Kohtz, D., and J. Leger, manuscript in preparation). The superstructure of the cultured cell types closely resembles that of cardiac myoblasts and primitive cardiac myocytes observed by electron microscopy during early embryonic development of the chicken heart (Hamburger-Hamilton stages 10–15; Manasek, 1970). These cells appear in vivo before the initiation of contractions and in the early functioning tubular heart, before cells with characteristics of fetal cardiac myocytes have developed.

Enriched myofibrillar proteins were prepared from cardiac myoblasts (HAM-1 cells were used) cultured in mitogen-rich or mitogen-poor media, and analyzed by SDS-PAGE. Myofibrillar proteins were not detected in cells cultured in mitogen-rich medium (Fig. 3, lane *C*). In contrast, bands corresponding to myosin heavy chain (200 kD) and actin (43 kD) were prominent in the preparations derived from cells cultured in mitogen-poor medium (Fig. 3, lane *B*). The proteins were transferred to nitrocellulose and blotted with monoclonal antibody MF-20, which reacts exclusively with sarcomeric isoforms of myosin heavy chain (Bader et al., 1982). A component of the 200-kD band reacted with MF-20, indicating the presence of an isoform of sarcomeric muscle myosin heavy chain (Fig. 3, lane *B'*).

Experiments were performed to assess the origin of cardiac myoblasts in cultures of dissociated fetal ventricle. Fetal cardiac cells were dissociated as described in Materials and Methods, and equivalent numbers of viable cells were plated directly in either mitogen-rich or mitogen-poor medium. Only minimal differences in the number of cells initially adhering to the plastic dishes was observed between the two types of media. After 0, 1, 3, 6, or 9 d in mitogen-poor medium, the cells were switched to mitogen-rich medium, and the time necessary for the cultures to reach confluence was recorded. As shown in Table I, preculturing the dissociated fetal ventricle cells severely inhibits the growth of cardiac myoblasts, such that after 6 d preculture in mitogen-poor medium cardiac myoblasts were not recovered. As will be detailed in the following sections, transcriptional activation of the ANF gene is a consistent phenotypic marker of differentiating cardiac myoblasts, regardless of their anatomical origin. The phenotype of the cells proliferating in mitogen-rich medium after preculturing 0, 1, 3, and 6 d in mitogen-poor medium was evaluated by inducing the cells to differentiate and comparing the levels of ANF transcription (Fig. 4). ANF transcription was observed in the cells derived from all four culture sequences (Fig. 4, lanes *a–d*), although the intensity of the ANF signal was reduced by preculturing in mitogen-poor medium.

One interpretation of the results presented in Table I is that cardiac myoblasts are derived directly from cells present in fetal cardiac tissue and not from cells expressing the phenotype of cultured fetal cardiac myocytes (the cells shown in Fig. 1 *a*). To estimate the percentage of dissociated fetal ventricle cells that eventually give rise to cardiac myoblasts, the following experiment was performed. A characterized cardiac myoblast strain (HAM-1) was plated in a series of different densities. Dissociated fetal ventricle cells were also plated at a series of different densities, and the time required for the cultures to reach confluence was recorded and compared (Table II). The data suggest that ~5% of the dissociated fetal ventricle cells generate cardiac myoblasts. Two interpretations are possible: a distinct subpopulation of fetal cardiac cells gives rise to cardiac myoblasts in culture; or, fetal cardiac myocytes (some of which are proliferating in vivo) may,



at a specific phase of their cell cycle, be susceptible to phenotypic conversion into cardiac myoblasts in culture.

The phenotypic stability of cardiac myoblasts was evaluated by comparing the induction of ANF stability in a strain of cardiac myoblasts passed one, three, six, and nine times (each passage represents a 1:10 dilution). As shown in Fig. 4, ANF transcription in differentiating cardiac myoblasts remains a stable feature of the cardiac myoblast phenotype for at least nine passages (30 generations). Independent experiments have indicated that cardiac myoblasts can be carried

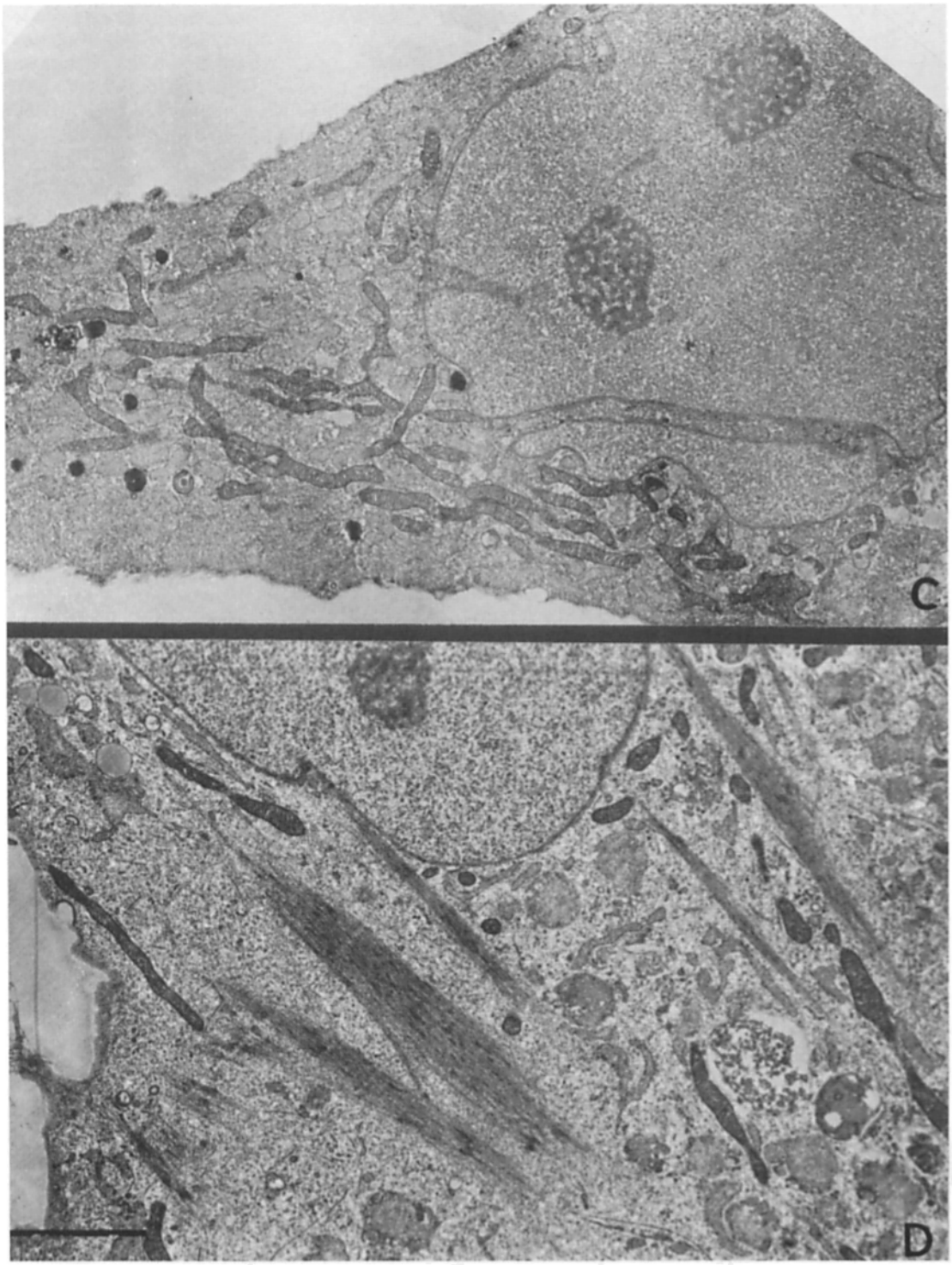


Figure 2. Phase-contrast and electron microscopy of human cardiac myoblasts. Human cardiac myoblasts (HAM-1 strain) were cultured in mitogen-rich medium (*A* and *C*) or mitogen-poor (*B* and *D*) medium for 5 d. The cells were subsequently examined by phase-contrast microscopy (*A* and *B*), or fixed and embedded for examination by electron microscopy (*C* and *D*). Arrowheads indicate cells in mitosis. Sections were cut directly from cells cultured on coverslips. Bars: (*A*) 30 μm; (*B*) 50 μm; (*C* and *D*) 1 μm.

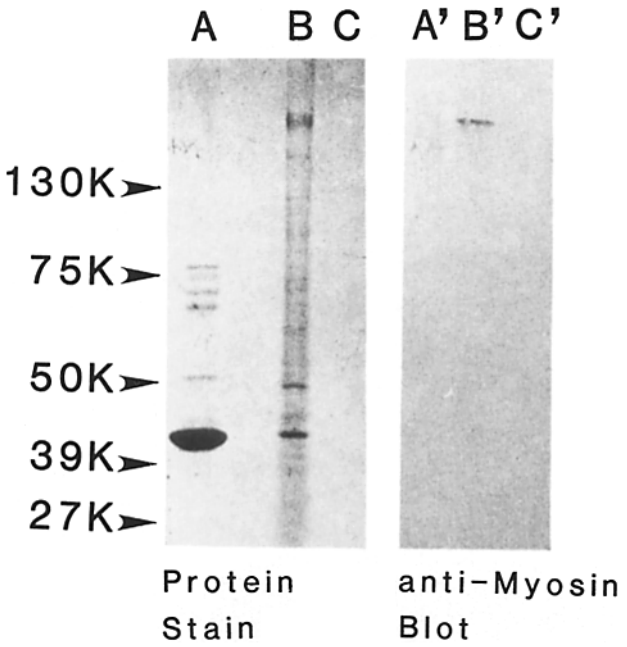


Figure 3. Myofibrillar proteins isolated from differentiated human cardiac myoblasts. Triton X-100–insoluble, high density myofibrillar proteins were isolated from human cardiac myoblasts (HAM-1 cells were used). Proteins were resolved on 5–15% SDS–polyacrylamide gels and stained with Coomassie blue (*A*, *B*, and *C*). Lane *A*, skeletal actin purified from rabbit back muscle (shown as a marker for actin); lane *B*, myofibrillar proteins isolated from cardiac myoblasts cultured in differentiation medium for 10 d. Myosin (200 kD) and actin (43 kD) constitute prominent bands; tropomyosin (34 kD) was not easily detected. Lane *C*, protein material isolated using the same isolation procedure and an equivalent number of cardiac myoblasts as used for lane *B*, except that these cells were cultured to confluence in growth medium. No Triton X-100–insoluble, high density structures were isolated. The samples (lanes *A'*, *B'*, and *C'*) were transferred to nitrocellulose and Western blotted with monoclonal antibody MF-20 (Bader et al., 1982). The 200-kD protein present in the differentiated cardiac myoblast myofibrillar proteins (lane *B*) reacted with MF-20, confirming its identity as an isoform of striated muscle myosin.

Table I. Effect of the Absence of Mitogens on the Generation of Cardiac Myoblasts

Culture conditions	Confluence	Phenotype
	d	
MR	11	d
1 d MP before MR	13	a
3 d MP before MR	17	b
6 d MP before MR	27	c
9 d MP before MR	–	–

Fetal ventricular cells were dissociated (see Materials and Methods) and plated in either mitogen-rich (*MR*) or mitogen-poor (*MP*) medium. 4,000 viable cells were used per experimental determination (35-mm culture dish). After 1, 3, 6, or 9 d in mitogen-poor medium, the cells were switched to mitogen-rich medium, and the number of days required for the cells to reach confluence was recorded. Description of the phenotypic marker used for these cultures (*a–d*) is given in Fig. 4. The cells that eventually arose from the cultures grown 9 d in mitogen-poor medium did not bear a morphological resemblance to cells considered as cardiac myoblasts.

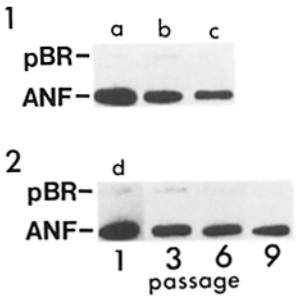


Figure 4. Effects of culture conditions and passaging on the phenotypic stability of cardiac myoblasts. As will be detailed in Figs. 6 and 7, induction of ANF transcription, as assayed by the generation of nuclear run-on transcripts, can be used as a differentiation marker of cardiac myoblasts. Cells were cultured as described in Table I (indicated as *a–d*), induced to differentiate by culturing 5 d in mitogen-poor medium, and tested for ANF transcription by nuclear run-on transcription assay. Run-on transcripts were analyzed by hybridization to ANF cDNA (a Pst I fragment of pGH33) and pBR322 (pBR; used as a control). The cells cultured in lane *d* were passed (1:10) one, three, six, and nine consecutive times, then induced to differentiate by culturing 5 d in mitogen-poor medium. ANF transcription was assayed by nuclear run-on transcription, and run-on transcripts were analyzed by hybridization to ANF cDNA clone and pBR322 (a control).

for at least 15 passages while maintaining a stable phenotype (not shown).

Differentiation of Human Cardiac Myoblasts Results in the Induction of ANF Expression

Expression of ANF has been observed at high levels in fetal atrium and ventricle (Bloch et al., 1986), and in situ hybridization experiments (Hamid et al., 1987) have revealed ANF transcripts in atrial and ventricular myocytes and not in other types of cells in the heart. Expression of ANF therefore represents a useful marker of cardiac myogenesis. These observations also suggest that induction of ANF expression could mark the differentiation of cardiac myoblasts into a cardiac myogenic lineage. To test this hypothesis, cardiac myoblasts were grown in mitogen-rich medium (0 d) or in differentiation medium for 3 or 7 d. Total cellular DNA was isolated from the cells and analyzed for ANF expression by Northern blotting (Fig. 5 *A*). All the cardiac myoblasts strains expressed low to undetectable levels of ANF mRNA when cultured in mitogen-rich medium. When cultured in mitogen-poor medium, A1 and RV-1 cells expressed high levels, HAM-1 cells expressed lower levels, and LV-1 cells expressed undetectable levels of ANF mRNA (Fig. 5 *A*). Expression of ANF mRNA marked the induction of differentiation by mitogen-poor medium in most of the cardiac myoblast cell strains. As a control, a strain of human fetal skeletal myoblasts (HUSK-1; Kohtz, D. S., unpublished observations) was cultured similarly and induced to differentiate in mitogen-poor medium. Expression of ANF was not observed in undifferentiated or differentiated HUSK-1 cells.

One of the cardiac myoblast strains (A1) producing high levels of ANF transcripts was analyzed in a more expanded time-course experiment (Fig. 5 *B*). ANF transcripts were detected 12–24 h after the induction of cardiac myoblast differentiation by mitogen-poor medium. In some cases (not shown here), ANF mRNA expression has been detected in cardiac myoblasts before introducing the cells into mitogen-poor medium. ANF expression in these cases appears to be dependent on depletion of the medium by confluent cells.

Table II. Evaluation of the Relative Number of Cells in Dissociated Fetal Ventricle That Generate Cardiac Myoblasts in Culture

HAM-1 cells	Confluence	Ventricular cells	Confluence
n	*d*	*n*	*d*
2,000	5	2,000	16
1,000	6	1,000	19
500	8	500	23
250	11	250	29
125	15	125	39
100	16	100	–
50	21	50	–
25	27	25	–

HAM-1 cardiac myoblasts and dissociated fetal ventricular cells were plated at the indicated starting density (cells per ml; 2 ml/35-mm culture dish) and allowed to reach confluence in mitogen-rich medium. The number of days required for the cultures to reach confluence was recorded, and by comparison, an inoculum of 100 HAM-1 cells was determined to be roughly equivalent to 2,000 dissociated fetal ventricular cells. Assuming that the rate of fibroblasts proliferation in these cultures is significantly slower than that of cardiac myoblast proliferation, these data suggest that ~5% of the dissociated fetal ventricular cells acquire the phenotype of cardiac myoblasts.

Transcriptional Regulation of ANF and Histone H3 Expression in Differentiating Cardiac Myoblasts

Nuclear run-on transcription analyses were used to assess the contribution of transcriptional mechanisms to the regulation of ANF and histone H3 expression in differentiating cardiac myoblasts. Transcriptionally active nuclei were isolated from cardiac fibroblasts (see Materials and Methods), HUSK-1 (skeletal myoblasts), HAM-1, LV-1, RV-1, or A1 cells cultured in either mitogen-rich or mitogen-poor medium for 5 d. Radiolabeled run-on transcripts from these nuclei were analyzed by hybridization to cloned histone H3, ANF, and 28-S ribosomal probes. In all the cell strains tested, histone H3 was transcribed at high levels in mitogen-rich medium and at significantly lower or undetectable levels in mitogen-poor medium. This is consistent with other studies indicating that histone H3 transcription is significantly higher in proliferating than in nonproliferating cells (Zhong et al., 1983; Heintz et al, 1983). Induction of ANF transcription was observed in the cardiac myoblast cells that were cultured in mitogen-poor medium for 5 d (Fig. 6). Induction of ANF transcription was not observed in human cardiac fibroblasts or human skeletal myoblasts (HUSK-1) cultured similarly (Fig. 6). From these results it can be concluded that transcriptional activation of the ANF gene occurs during differentiation of cardiac myoblasts into primitive cardiac myocytes.

Time-course, run-on transcription experiments were performed to determine the kinetics of histone H3 and ANF transcriptional regulation. HAM-1 cells were cultured in mitogen-rich medium (0 d), or in mitogen-poor medium for 1, 2, 3, or 5 d. Nuclear run-on transcription assays indicated that histone H3 transcription is reduced 1 d after the cells are switched to mitogen-poor medium. Transcription of ANF increases for 3 d, reaching its plateau at 5 d (Fig. 7). Whether this pattern of expression is associated with increases in the rate of transcriptional initiation in these cells, or with a gradual increase in the number of nuclei transcribing ANF over the 5-d period cannot be determined from these data. When HAM-1 cells were cultured at confluence in mitogen-poor medium supplemented with chick embryo extract (DME + 4% horse serum + 1% chick embryo extract), transcriptional activation of the ANF gene was attenuated, despite the same decrease in histone H3 transcription as was observed with mitogen-poor medium alone (Fig. 7; 1′, 2′, 3′, and 5′ d). This result suggests that inhibition of cellular proliferation alone is not sufficient to induce differentiation of cardiac myoblasts.

Contributions of Posttranscriptional Mechanisms of Regulation to ANF Expression During Cardiac Myoblast Differentiation in Culture

Although all the cardiac myoblast strains were induced to transcribe ANF by mitogen-poor medium, the levels of ANF mRNA in the different strains varied significantly. In particular, strains LV-1 and HAM-1 transcribed ANF in the run-on assay at levels equivalent to A1 and RV-1 (Fig. 6), while the level of ANF mRNA in LV-1 and HAM-1 cells was significantly lower than in the other strains (Fig. 5). This implies that posttranscriptional mechanisms may contribute to the regulation of ANF expression during cardiac myoblast differentiation. From the data obtained so far, the anatomical origin of the cardiac myoblasts appears to correlate to the relative stability of ANF transcripts: transcripts are more stable in strains derived from right atrium (A1) and right ventricle (RV-1), and less stable in strains derived entirely (LV-1) or in part (HAM-1) from left ventricle. This result indicates that while cardiac myoblasts represent an extremely primitive myocardial phenotype, some divergence between cells derived from different anatomical regions of the heart may already be detected.

To assess further the role of posttranscriptional mechanisms on the modulation of ANF expression, differentiating cardiac myoblasts were cocultured with cells of neuronal origin. Several studies (Seidler and Slotkin, 1979; Slotkin et al., 1980; Kirby et al., 1983; Stewart et al., 1986) have indicated that neural crest cells from the neural folds adjacent to somites 1–4 influence cardiac growth in a manner that is dependent on the developmental stage of the heart. With this in mind, nuclear run-on assays were conducted with differentiating HAM-1 cells cocultured with SH-SY5Y neuroblastoma cells (Perez-Polo et al., 1979). The two cell types were cultured in chambers separated by either 3.0-μm or 0.45-μm polycarbonate membranes. SH-SY5Y cells were cultured in the upper chamber and laid directly on the HAM-1 cells cultured in petri-style dishes. Transcriptionally active nuclei were isolated from HAM-1 cells cultured in mitogen-rich medium alone, or cocultured in mitogen-poor medium for 1, 2, 3, or 5 d. Radiolabeled run-on transcripts from these nuclei were hybridized to cloned probes for ANF and histone H3 (Fig. 7). No significant differences were noted between the time course of ANF transcription of HAM-1 cells cultured alone and HAM-1 cells cocultured with SH-SY5Y cells in mitogen-poor medium.

Cytoplasmic RNA was isolated from the cytosol fractions of the same cocultured cells as nuclei were extracted from for the run-off transcription experiments. The RNA was resolved by formaldehyde-agarose gel electrophoresis, then blotted with a complete cDNA probe for ppANP (pGH33), and a highly specific probe for cardiac actin derived from the 3′ untranslated region of the cDNA (Bains et al., 1984).

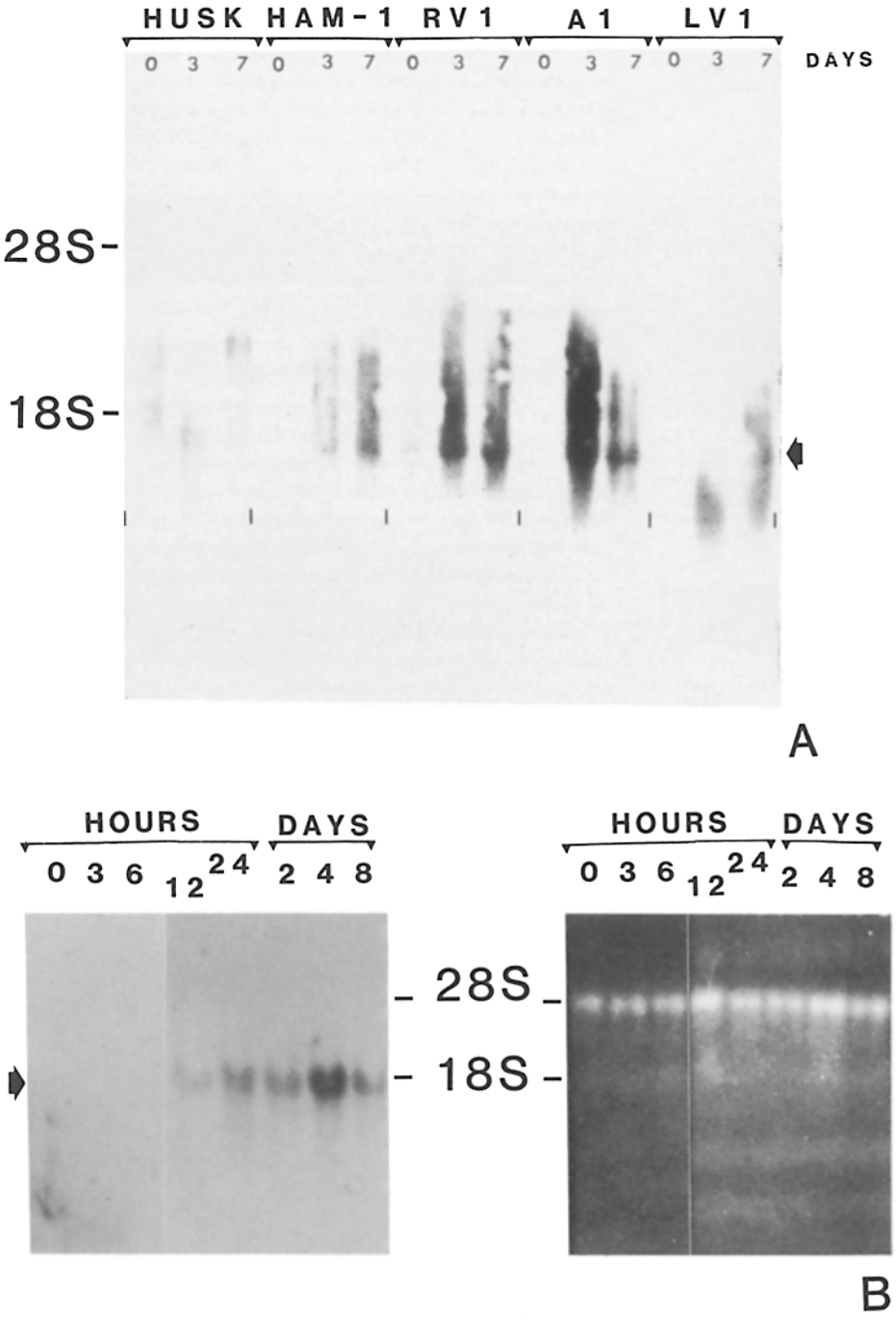


Figure 5. Expression of ANF mRNA by differentiating human cardiac myoblasts. (*A*) Expression of ANF by cultured human cardiac myoblast strains HAM-1, RV-1, A1, and LV-1 was monitored by Northern blot analysis. HUSK-1 human fetal skeletal myoblasts (Kohtz, D., unpublished observations) are shown as a control. Cells were cultured either in mitogen-rich medium (0 d), or in mitogen-poor medium for 3 or 7 d to induce differentiation. Total cellular RNA was isolated from the cells at each time point, and 20 μg was loaded per lane. Arrow indicates the position of ANF mRNA. (*B*) An expanded time course experiment was conducted with the A1 strain of cardiac myoblasts. Cells were cultured in mitogen-rich medium (0 d), then switched to mitogen-poor medium for 3, 6, 12, or 24 h, and 2, 4, or 8 d. Total cellular RNA was isolated from the cells at each time point, and 10 μg was loaded per lane. Arrow indicates the position of ANF mRNA. On the right is a gel run in parallel (samples were split) stained for RNA with acridine orange (shown as a loading control).

Differentiating HAM-1 cells cultured in the absence of neuroblastoma cells accumulated no detectable ANF mRNA, while cardiac α-actin mRNA steadily accumulated over the 5-d period (Fig. 8). Differentiating HAM-1 cells cocultured with neuroblastoma cells through 45-μm filters contained detectable ANF mRNA on day 5. Differentiating HAM-1 cells cocultured with neuroblastoma cells through 3.0-μm pore membranes contained high levels of ANF mRNA at the earliest time assayed (day 1). The maximum level of ANF mRNA in these cultures was observed on day 2, after which it decreased slightly (days 3 and 5; Fig. 8). The data indicate that the presence of cocultured neuroblastoma cells affects the posttranscriptional processing of ANF mRNA in differentiating HAM-1 cells. This effect was more apparent when 3.0-μm pore membranes were used to separate the cells than when 0.45-μm pore membranes were used. As both types of membrane allow rapid diffusion of secreted molecules, the accentuation of ANF mRNA stability probably was not induced by a small soluble factor (such as neurotransmitter) secreted by the neuronal cells.

Cardiac α-actin transcripts were detected at low levels in replicating cardiac myoblasts, and at higher levels in the differentiating cells (Fig. 8). Although the levels of cardiac α-actin mRNA were consistently higher in differentiating

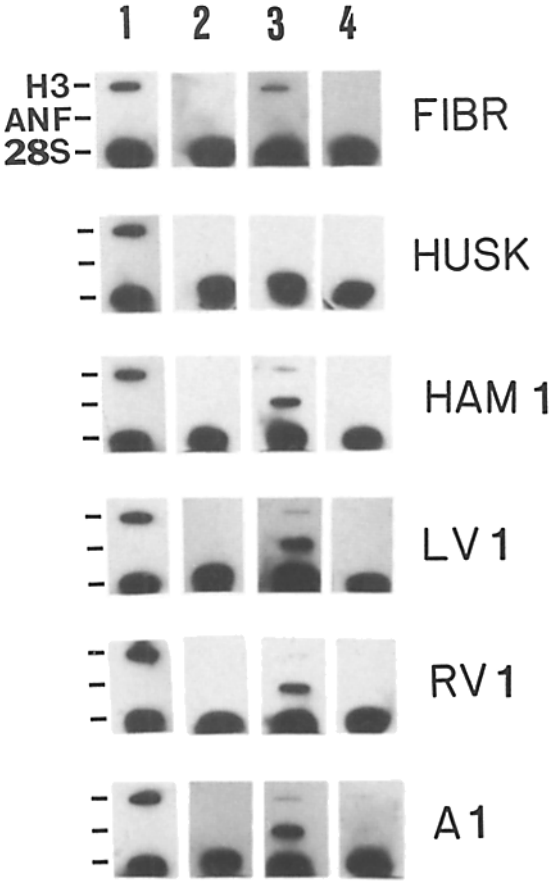


Figure 6. Nuclear run-on transcription analyses of ANF, histone H3, and 28-S ribosomal RNA expression in human cardiac myoblasts. Human cardiac myoblast strains (HAM-1, LV-1, RV-1, and A1), human skeletal myoblasts (*HUSK*), and human cardiac fibroblasts (*FIBR*) were cultured in mitogen-rich medium (columns *1* and *2*) or in mitogen-poor medium (columns *3* and *4*) for 5 d. Transcriptionally active nuclei were isolated, and radiolabeled run-on transcripts were hybridized to cloned cDNA probes for histone H3, ANF, and 28-S ribosomal RNA (see Materials and Methods). Run-on transcription assays were performed in the presence (columns *2* and *4*) or absence (columns *1* and *3*) of 2 μg/ml α-amanitin.

cardiac myoblasts than in replicating cells, considerable variation was observed between independent cultures of differentiated cells (Fig. 8). The factors influencing the expression of cardiac α-actin mRNA in differentiating cardiac myoblasts will be better defined in a future report.

Discussion

The generation of a culture model for early myocardial development presented two technical problems: the choice of appropriate conditions for growth of the cardiac myoblasts, and the choice of appropriate conditions to induce differentiation of the cells. The medium used in these experiments for the growth of cardiac myoblasts, although complex, contains no unexpected components. Basic FGF is a mitogenic factor for virtually all cells of mesodermal origin (Gospodarowics et al., 1976). MSA has been shown to be an analog for somatomedin A and a mitogenic factor for embryonic skeletal muscle cells (Ewton and Florini, 1980). High concentrations of fetal bovine serum and chick embryo extract are routinely used to promote the growth of skeletal myoblasts (Spizz et al., 1986). Higher concentrations of FGF and MSA (200 ng/ml) eliminated the need for chick embryo extract in the mitogen-rich growth medium, although the extract alone at high concentrations did not efficiently promote the growth of A1, LV-1, or RV-1 cells. Some strains of cardiac myoblasts have been derived in medium containing 1% chick embryo extract in the absence of exogenously added FGF and MSA; however, these strains grew very slowly and are prone to fibroblast overgrowth. None of the strains of cardiac myoblasts proliferated in 20% fetal bovine serum alone. The development of a fully defined growth medium for cardiac myoblasts is in progress.

The choice of a differentiation medium was clearly dependent on determining a marker for differentiation of the cardiac myoblasts. Atrial natriuretic factor is expressed at very high levels in fetal atrium and ventricle (Block et al., 1986; Kikuchi et al., 1987), and is restricted to myocardial cells in the heart (Hamid et al., 1987). Induction of ANF expression was identified in this study as a marker for the differentiation of cardiac myoblasts in culture. A mitogen-poor

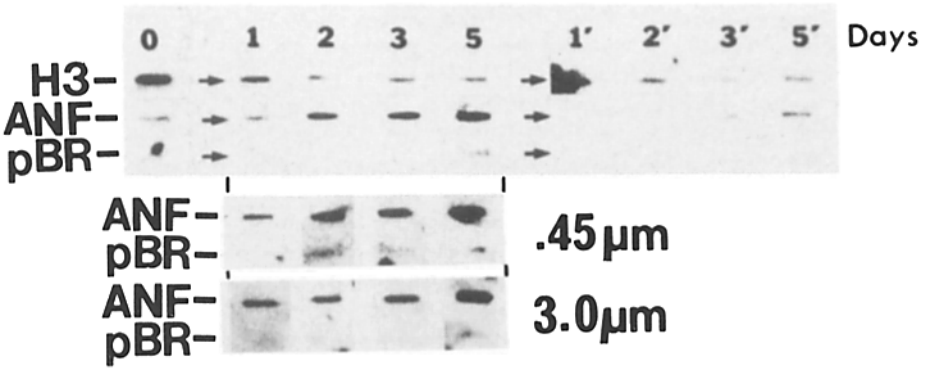


Figure 7. Time-course nuclear run-on transcription analyses of ANF expression by differentiating cardiac myoblasts. Human cardiac myoblasts (HAM-1 cells were used) were grown to 90% confluence in mitogen-rich medium (0 time). The medium was then changed to mitogen-poor medium. At the times indicated (1, 2, 3, and 5 d) transcriptionally active nuclei were isolated from the HAM-1 cells. Run-on transcription assays were performed, and the transcripts were analyzed by hybridization to the following probes (plasmids described in Materials and Methods): *H3*, histone h3; *pBR*, pBR322; and ANF. HAM-1 cells were cultured in mitogen-poor medium without SH-SY5Y cells; transcriptional activation of the ANF is evident. HAM-1 were cells cultured in mitogen-poor medium supplemented with 1.0% chick embryo extract (indicated as days 1′, 2′, 3′, and 5′); transcriptional activation of the ANF gene is attenuated. HAM-1 cells cultured in mitogen-poor medium with SH-SY5Y neuroblastoma cells separated by a 0.45-μm or a 3.0-μm pore polycarbonate membrane (as indicated).

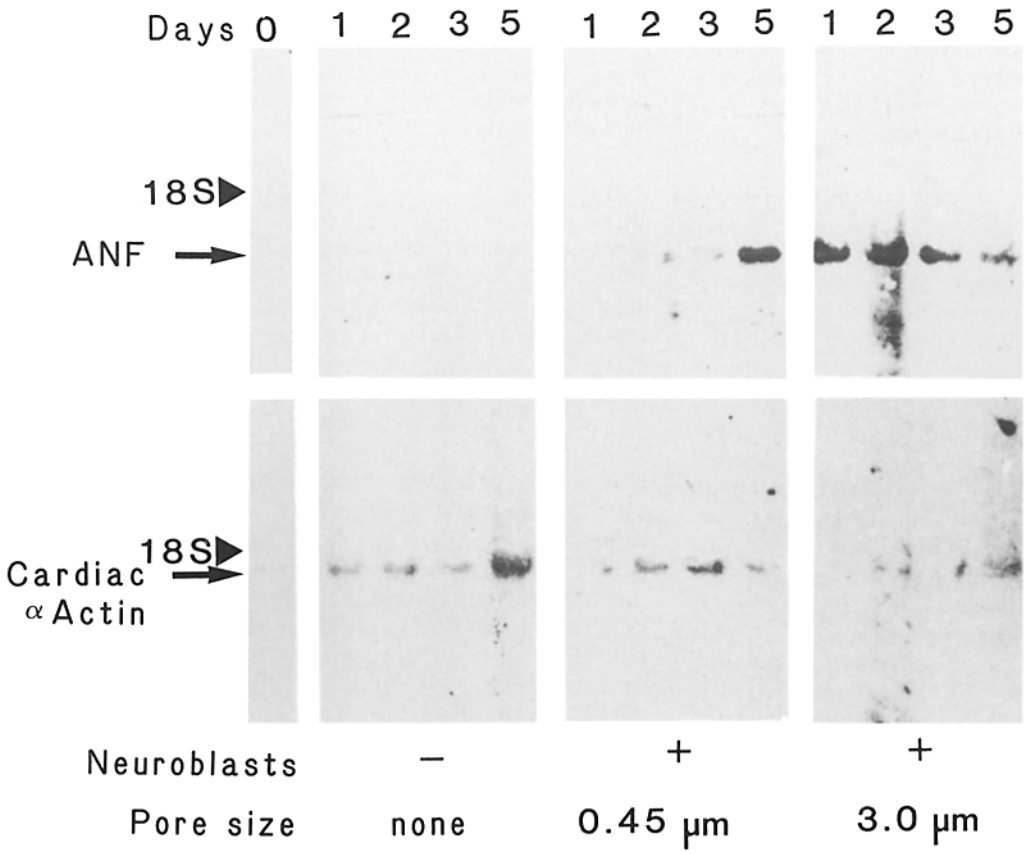


Figure 8. Northern blot analysis of ANF and cardiac α-actin mRNA expression in cardiac myoblasts induced to differentiate in the presence of neuronal cells. The cytosol fractions from some of the cells used for the run-off transcription experiments shown in Fig. 7 were retained, and total cytoplasmic RNA was isolated. RNA was resolved on 1.5% formaldehyde/agarose gels, transferred to GeneScreen plus (New England Nuclear), and hybridized to either a full length cDNA probe for ANF (pGH33) or the highly specific 3′ untranslated region of the cardiac actin gene (Bains et al., 1984). ANF mRNA is slightly >1 kb, while cardiac α-actin is slightly >1.3 kb. The number of days in differentiation medium, the presence or absence (+ or −) of cocultured SH-SY5Y cells, and the pore size of the membranes separating the two types of cells is indicated. ANF mRNA is only present in HAM-1 cells cocultured with neuroblasts, and increases earlier with the use of larger pore (3.0 μm) filters. *18S* refers to the position of 18-S ribosomal RNA.

medium (DME + 4% horse serum) was used to induce differentiation of the cells, similar to that used to induce the differentiation of skeletal myoblasts (Clegg et al., 1987). Expression of ANF marked the differentiation of three (A1, RV-1, and HAM-1) of the four cardiac myoblast cell strains tested. Induction of ANF expression in these strains by mitogen-poor medium resulted from transcriptional activation of the ANF gene. In one of the four strains tested (LV-1), transcriptional activation of the ANF gene was observed, but stable transcripts did not accumulate in the cells.

Strains of cardiac myoblasts were derived from different anatomical regions of the fetal heart. When induced to differentiate, strains derived from right atrium (A1) and right ventricle (RV-1) accumulated high levels of ANF transcripts; a strain derived from total ventricle accumulated a significantly lower level of ANF transcript; while a strain derived from left ventricle (LV-1) did not accumulate detectable ANF transcripts. These observations reflect variations in posttranscriptional processing of the ANF transcripts, since run-on transcription analyses indicated that the transcriptional activity of the ANF gene is equivalent in the four cell strains. Coculture of two of the strains (LV-1 [data not shown] and HAM-1) with neuroblastoma cells significantly accentuated their accumulation of ANF transcripts during differentiation, also through a mechanism involving posttranscriptional regulatory events. The results suggest that the pathways of differentiation for cardiac myoblasts from different anatomical regions of the heart are not identical even at this early phase of differentiation, and that a variety of signals may modulate their development. Evidence is also presented here that posttranscriptional mechanisms contribute to the development of phenotypic heterogeneity among differentiating cardiac myoblasts.

While the stimuli inducing ANF secretion have not been completely defined, in vitro studies have shown that adrenaline, argenine vasopressin, acetylcholine, and atrial distension all promote its release (Sonnenberg and Veress, 1984). Some studies have indicated that cells of the sympathetic nervous system, in particular those of the right atrial appendage, may mediate the induction of ANF release. Surgical isolation of the heart from the sympathetic nervous system reduces the natriuresis associated with hypervolemia (Veress and Sonnenberg, 1984), a result which has been recently attributed to impaired release of ANF (Garcia et al., 1987). While the results presented here do not discount the possibility that the sympathetic nervous system can influence ANF release by

regulating its secretion, an alternative (and compatible) role for these cells in the regulation of ANF expression is suggested. The experiments described in this report suggest that the innervation of cardiac myocytes can influence their ability to synthesize ANF by a mechanism that involves the posttranscriptional regulation of ANF mRNA stability.

Extracellular matrix components have been shown in other culture systems to influence the posttranscriptional regulation of gene expression at the level of mRNA stability (reviewed by Reid et al., 1988). Similarly, the effects of cocultured neuronal cells on the accumulation of ANF transcripts in differentiating cardiac myoblasts may be attributed to extracellular matrix components secreted by the neuronal cells. Experiments in progress are aimed at deciphering the role of various extracellular matrix components on cardiac myoblast differentiation.

The low myosin to actin ratio (0.31 as determined by microdensitometry scanning) of the differentiated cardiac myoblast myofibrillar proteins reflects the primitive phenotype of the cells. Myofibrillar proteins have been examined in heart tissues at various phases of the development (Lemanski et al., 1976). Before the initiation of beating, myofibrillar proteins isolated from primitive cardiac myocytes contain a low myosin to actin ratio (0.20–0.33) and little tropomyosin; after beating has initiated, the myosin to actin ratio exceeds 0.50, and the mass of tropomyosin in the myofibrillar proteins becomes significant. Using the present culture conditions, neither differentiated nor undifferentiated cardiac myoblasts display spontaneous contractions. These observations indicate that cardiac myoblasts in culture execute a differentiation program that is executed by cardiac myoblasts in vivo before the initiation of spontaneous contractions. These cells then disappear shortly after the appearance of a functioning tubular heart (Manasek, 1970).

Cultured human cardiac myoblasts, when changed from a mitogen-rich to a mitogen-poor medium, differentiate into cells that represent an intermediate in myocardial development. Elucidation of the factors responsible for inducing the further differentiation of these primitive cardiac myocytes into fetal or adult cardiac myocytes is the focus of current investigations. Further studies should eventually be possible of the epiphenomenology associated with the development of local and anatomical divergence among cardiac myocytes, as well as of the molecular events which manifest these changes.

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