Differentiation of Cardiac Myocytes after Mitogen Withdrawal Exhibits Three Sequential States of the Ventricular Growth Response

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Abstract. During cardiac myogenesis, ventricular muscle cells lose the capacity to proliferate soon after birth. It is unknown whether this developmental block to mitotic division and DNA replication might involve irreversible repression of the cellular oncogene c-*myc*. Ventricular myocytes from 2 d-old rats continued to differentiate in vitro during 15 d of mitogen withdrawal, as shown by the formation of cross-striations, increased proportion of the muscle isoenzyme of creatine kinase, stable expression of α-cardiac actin and myosin heavy chain mRNAs, and appropriate down-regulation of α-skeletal actin mRNA. After mitogen withdrawal for 2 d, serum evoked both DNA synthesis and mitotic division; after 7 d, DNA replication was uncoupled from cell division; after 15 d, DNA synthesis itself was markedly attentuated. These three distinct phenotypic states resemble the sequential properties of growth found in the neonatal rat heart in vivo. Despite failure to induce DNA replication or division after 15 d of mitogen withdrawal, serum elicited both c-*myc* and α-skeletal actin as found during hypertrophy of the intact heart. The results agree with previous evidence that one or more functional pathways that transduce the effects of serum factors may persist in older cardiac muscle cells, and indicate that irreversible down-regulation of c-*myc* cannot be the basis for the loss of growth responses.

IDENTIFYING the cellular mechanisms that control cardiac muscle growth is essential to understanding cardiac myocytes' failure to regenerate after injury, as well as hypertrophy triggered by a physiological load. Fundamental differences may distinguish cardiac development from that of skeletal muscle cells or other readily accessible systems. First, proliferation of skeletal myoblasts is mutually exclusive with activation of muscle-specific genes (9, 29, 38), whereas cardiac myocytes synthesize muscle-specific proteins and become contractile while still proliferating (35). Second, formation of binucleated myocytes during the loss of growth capacity in cardiac muscle (karyokinesis uncoupled from cytokinesis) differs importantly from assembly of multinucleate myotubes via cell fusion (6). Third, adaptive growth of cardiac muscle involves enlargement (hypertrophy) and DNA synthesis (nuclear hyperplasia) in pre-existing muscle cells (26), while skeletal muscle growth is mediated in part by recruitment of "satellite" precursor cells. Finally, increased contractile work induces cardiac muscle to reexpress genes that encode "embryonic" contractile and cytosolic proteins (18, 41) but can accelerate the maturation of myosin heavy chain isoforms in skeletal muscle (19). These observations suggest that cardiac myocytes have unique biological features and that distinct molecular mechanisms may control their proliferative growth as well as the onset and establishment of a differentiated cardiac phenotype. However, the exact cellular and molecular mechanisms that regulate development in cardiac myocytes remain to be elucidated.

Knowledge of the processes that regulate growth and differentiation has been expanded by the discovery that cellular oncogenes may encode peptide mitogens and their receptors or convey growth factor signals from the surface membrane to the nucleus (reviewed in reference 38). Intranuclear oncogene proteins such as *fos*, *erb*A, and *jun* bind cis-acting regulatory DNA sequences and may affect cell behavior directly. The impact of growth factors on proliferation is thought to require, in particular, the proto-oncogene, c-*myc* (7). In quiescent skeletal muscle as in other lineages, c-*myc* gene expression is rapidly increased by growth factors (46); conversely, down-regulation of c-*myc* is an early event during myogenic differentiation (9, 40) which fails to occur in muscle cell lines that cannot differentiate (32, 42). During normal cardiac development, c-*myc* becomes down-regulated in ventricular tissue (39) but can be elicited by interventions that provoke hypertrophic growth in vivo and in vitro, such as aortic constriction and norepinephrine, respectively

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(18, 27, 48). The putative function suggested by these temporal relationships has been substantiated, at least in nonmuscle cells, through the use of bacterially produced *myc* protein or deregulated *myc* genes as surrogates for growth factors (reviewed in reference 38) and by anti-*myc* antibodies (17) and anti-sense oligonucleotides (51) that block DNA replication or trigger differentiation. Although down-regulation of c-*myc* is not obligatory for muscle development, autonomous expression of c-*myc* can delay or partially inhibit the myogenic phenotype (3, 40). In contrast, the viral *myc* gene can abolish differentiation of skeletal muscle cells, most likely by maintaining autonomous proliferation (12). DNA sequences recognized by *myc* are found at or near the initiation site of DNA replication (17). Recently, v-*myc* also was shown to impair cardiac differentiation and produce cardiac rhabdomyosarcomas in avian embryos (37).

Together, these observations suggest that c-*myc* is intimately associated with cardiac muscle cell growth and raise the hypothesis that a block to mitogenic induction of c-*myc* might provide a basis for the age-dependent loss of cardiac growth capacity. As a first step to determine whether cardiac growth control involves developmental regulation of specific components of the machinery for growth factor signal transduction, we have isolated ventricular cardiac myocytes from 2-d postnatal rats and examined their developmental properties during 2 wk of mitogen withdrawal. The uncoupling of DNA replication from mitotic growth followed by attenuation of DNA synthesis, corresponded to the phases of cardiac growth found in neonatal ventricular muscle (6, 30). Cardiac myocytes which neither replicate DNA nor divide retained at least partial responsiveness to serum, as demonstrated by induction of c-*myc* and the "embryonic" α-skeletal actin gene, as found after the transition to hypertrophic growth in vivo.

Materials and Methods

Cell Culture

Primary cultures of cardiac myocytes were prepared from the ventricles of 2-d-old Sprague-Dawley rats by 4 cycles of enzymatic digestion with gentle mechanical disaggregation. Cells were dissociated at 37°C and 40 rpm in Ca^{2+}- and Mg^{2+}-free PBS, supplemented with 1% glucose, 0.1% trypsin (TRL3), 0.1% collagenase (CLS), and 0.025% DNase I (D; Worthington Biochemical Corp., Freehold, NJ). After the final cycle of digestion, the dissociated cells were pooled in medium (DME; Ham's nutrient mixture F12[Gibco Laboratories, Grand Island, NY], 1:1, adjusted to 17 μM Hepes, pH 7.4, 3 μM $NaHCO_3$, 2 μM L-glutamine, and 50 $\mu g/ml^{-1}$ gentamicin) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT). The cell population was enriched for cardiac myocytes and partially depleted of cardiac mesenchymal cells by differential adhesiveness. The residual nonattached cells were plated at $\sim 10^5$ cells/cm^2 on polystyrene culture dishes treated with 0.1% gelatin (ICN Biochemicals Inc., Irvine, CA). After 12–16 h, the cells were maintained in serum-free medium supplemented with 1 μg/ml insulin, 5 μg/ml transferrin, 1 nM LiCl, 1 nM Na_2SeO_4, 25 μg/ml ascorbic acid, and 0.1 nM thyroxine. From day 4 onward, the medium also contained 0.5% calf serum (HyClone) and was replaced every 3 d. Primary cultures enriched for cardiac mesenchymal cells were obtained from the rapidly adherent fraction of ventricular cells.

cDNA Probes

To quantitate steady-state levels of c-*myc* mRNA, a 2.5-kbp XbaI–HindIII restriction fragment was isolated from plasmid pSVc-*myc*-1, containing exons 2 and 3 of murine c-*myc* (20). Glyceraldehyde-3-phosphate dehydrogenase (*gad*)[1] mRNA was measured using the 1.65-kbp HhaI restriction fragment of pGAD-28 (8). DNA fragments were labeled with α-^{32}P-dCTP (3,000 $Ci/mmol^{-1}$; Amersham Corp., Arlington Heights, IL) to 1–5 × 10^9 cpm/μg by the random primer method (13). Synthetic oligonucleotides used to examine rat α-cardiac and α-skeletal actin expression corresponded to the specific 3′ untranslated sequences 83–102 (25) and 48–67 (43), respectively. Probes were labeled at the 5′ end with α-^{32}P-ATP using T4 polynucleotide kinase, to a specific activity of 4–6 × 10^8 cpm/μg (24).

Northern Blot Hybridization

Total cellular RNA was isolated, size fractionated by formaldehyde–agarose gel electrophoresis (10 μg/lane), and transferred to nitrocellulose filters as previously described (32). For *myc* and *gad* cDNA probes, blots were washed in 2× SSC, 0.5% SDS for 45 min at room temperature and in 0.1× SSC, 0.1% SDS for 90 min at 60°C. For oligonucleotide probes, blots were washed for 45 min in 5× SSC at room temperature and for 90 s in 5× SSC at 55°C. Blots were exposed to Kodak XAR-2 film at −70°C with intensifying screens (Lightning-Plus; DuPont Co., Wilmington, DE) and were quantitated by scanning densitometry. Band intensity was linear with respect to RNA concentration.

Results

Mitogen Withdrawal Elicits Differentiated Structural and Biochemical Properties in Cultured Ventricular Myocytes

Cardiac cells formed a functional syncytium, as indicated by synchronous spontaneous contractions, from day 2 onward. By days 2 and 3, the contractile myocytes developed a characteristic morphology consisting of a coarse granular cytoplasm with dense inclusions and a dense round nucleus. By the criteria of morphology and spontaneous contraction, the ventricular myocytes were readily distinguished from nonmuscle cells (<10%), which exhibited a pale cytoplasm, thin filopodia, and a larger, ellipsoid nucleus bounded by a thin nuclear membrane. These respective properties were maintained through day 15 of mitogen withdrawal (cf. 5, 44). Cross-striations and binucleated ventricular muscle cells were found from day 6 or 7 onward. To examine whether the cardiac cells were stably differentiated after mitogen withdrawal, the distribution of CK isoenzymes was analyzed at day 2, 7, and 15 (11). The proportion of the "muscle" homodimer (*MM*) increased progressively, from 53% at day 2 to 59% at day 7, and, finally, to 70% at day 16 (Fig. 1, *A–C*). In contrast, the proportion of the "brain" homodimer (*BB*), which is relatively abundant both in immature cardiac myocytes and in nonmuscle cells (Fig. 1 *D*), was initially expressed at 11% and was stably maintained.

To define more completely the extent of differentiation in this in vitro model, expression of representative developmentally regulated sarcomeric proteins was analyzed by Northern hybridization. As shown in Fig. 2, steady-state levels of α-MHC mRNA did not deteriorate during 15 d of serum withdrawal (cf. 28, 52). In aggrement with its persistence at 14 d of age in vivo (22), β-MHC mRNA also was expressed in the oldest cells examined here. As discussed more fully below, basal expression of α-cardiac and α-skeletal actin mRNA during 2 wk in monolayer culture was, likewise, appropriate to the cells' developmental stage.

1. *Abbreviations used in this paper*: CK, creatine kinase; *gad*, glyceraldehyde 3-phosphate dehydrogenase; MHC, myosin heavy chain.

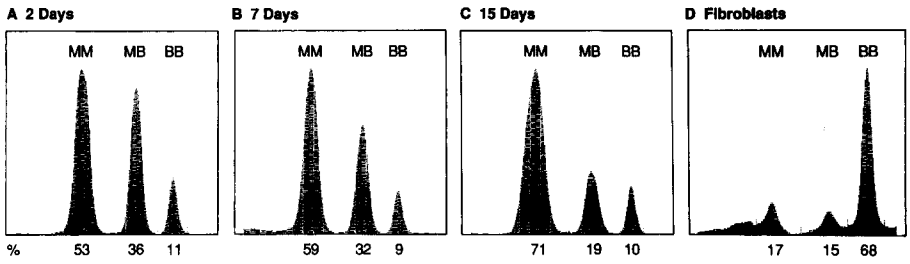

Figure 1. Distribution of CK isoenzymes in cultured cardiac myocytes. The dimeric isoenzymes (*MM*, *MB*, and *BB*) of creatine kinase were analyzed by agarose electrophoresis of cell lysates of cardiac myocytes cultured in differentiation medium for (*A*) 2, (*B*) 7, or (*C*) 15 d. The percentage of total CK activity comprising each isoenzyme is shown below the respective panel of the figure. (*D*) Distribution of CK isoenzymes in cultures enriched for cardiac mesenchymal cells after mitogen withdrawal for 7 d. CK isoenzymes were separated by agarose gel electrophoresis and measured by scanning fluorimetry of the creatine phosphate-dependent fluorescence generated as NADPH, using the coupled enzyme method of Rosalki (34).

Three Phases of Cardiac Growth Identified In Vitro Correspond to the Postnatal Development Observed in Intact Ventricular Muscle

To determine whether a time-dependent loss of growth capacity could be observed in isolated cardiac myocytes in the absence of hemodynamic and neurohumoral changes, quiescent ventricular muscle cells were challenged with serum mitogens (20% FBS) after 2, 7, or 15 d in differentiating medium, and were examined for cell number and [^{3}H]thymidine incorporation into DNA (Fig. 3). Initially, after 2 d of mitogen withdrawal, serum mitogens could provoke both DNA replication and subsequent mitosis (*left graph*). After the mitogenic challenge, [^{3}H]thymidine incorporation increased within 12 h and reached a peak at 21 h, after which cytokinesis occurred: the cell population increased 1.8-fold within 30 h. In contrast, after 7 d in differentiating medium, DNA synthesis became uncoupled from mitotic division: no increase in cell number was found, although DNA synthesis was evoked by serum to almost the same extent (*middle graph*). Even the capacity to replicate DNA in response to serum mitogens was markedly attenuated in ventricular myocytes that had been cultured in differentiating medium for 15 d (*right graph*). The incorporation of [^{3}H]thymidine into DNA was approximately one-fifth of that observed at day 2. No secondary peak of thymidine incorporation was detected to indicate prolongation of the G1 phase of the cell cycle, even with observation for up to 48 h after serum addition.

c-myc mRNA Remains Inducible in Cardiac Myocytes that Cannot Replicate DNA or Divide in Response to Serum Mitogens

To examine the hypothesis that irreversible repression of the c-*myc* gene in ventricular myocytes might provide a molecular basis for the age-dependent loss of cardiac growth capacity, the inducibility of c-*myc* mRNA in response to serum mitogens was determined in cardiac myocytes at each of the three sequential stages of growth response described above. In cardiac myocytes cultured for 2 d, which respond to serum with both DNA synthesis and proliferation, c-*myc* mRNA was induced by mitogens within 1 h, reached maximal induction at ~2 h, and returned to baseline levels between 12 and 24 h after serum addition (Fig. 4; data at 0.5–2 h, not shown), as in other myogenic cells (9, 46). Despite the sequential block to cytokinesis and even DNA synthesis in cardiac myo-

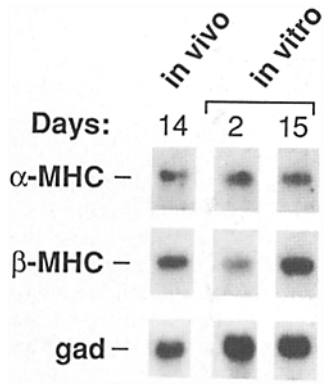

Figure 2. Expression of α- and β-MHC mRNA at 2 and 15 d of serum withdrawal. Total cellular RNA was isolated at the intervals indicated, and the levels of α- and β-MHC mRNAs were determined by Northern blot hybridization to ^{32}P-labeled synthetic oligonucleotide probes (14). As the specific activities and exposure times for autoradiography differed, the signal intensities are not intended to represent the relative abundance of the two transcripts. Levels of *gad* mRNA did not change significantly. Control 14-d rat cardiac mRNA is shown at the *left*.

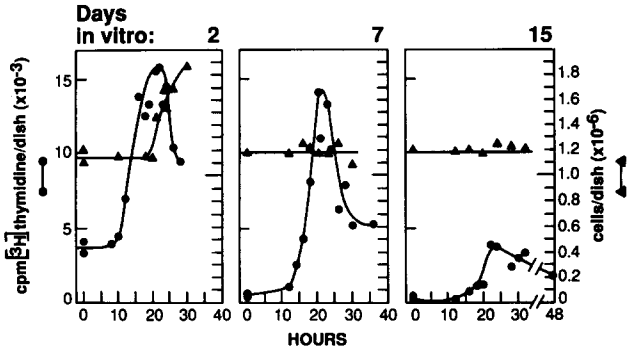

Figure 3. The age dependence of [^{3}H]thymidine incorporation and cell proliferation after exposure of quiescent cardiac myocytes to serum mitogens identifies three distinct stages of cardiac growth capacity. Cardiac myocytes were cultured in differentiation medium for (*left graph*) 2, (*center graph*) 7, or (*right graph*) 15 d and were then transferred to medium containing 20% FBS at time 0. (●) Acid-insoluble incorporation of [^{3}H]thymidine was determined after incubation for 60 min at 37°C in serum-free medium containing 3 μCi/ml [^{3}H]thymidine. (▲) Cell number was determined in duplicate using a hemocytometer. Similar data were observed in each of three independent experiments.

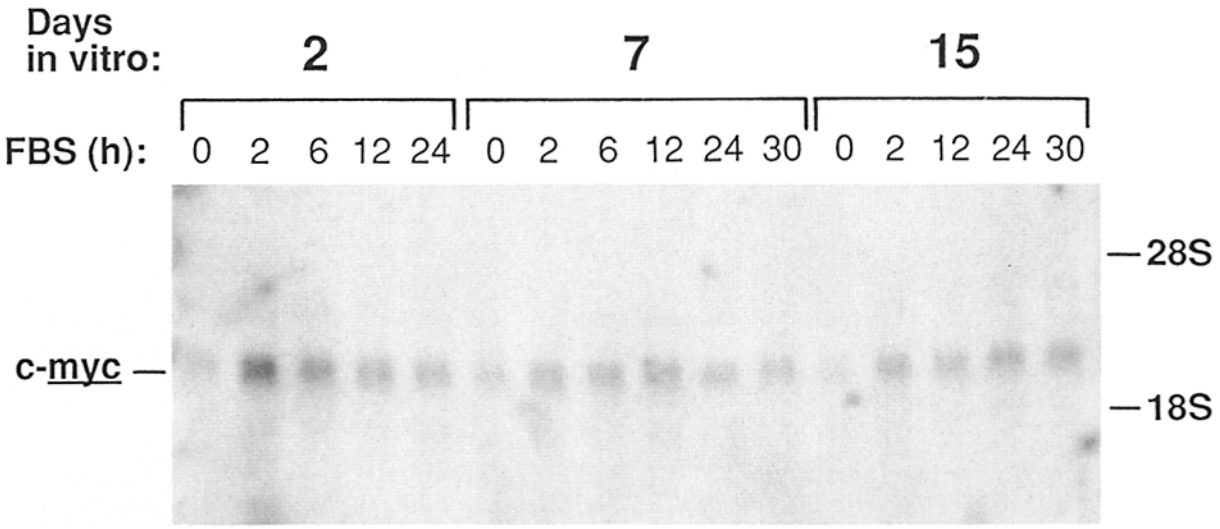

Figure 4. Irreversible repression of c-*myc* is not required for the loss of cardiac growth capacity. Cultured cardiac myocytes were maintained in differentiation medium for 2, 7, or 15 d and were then transferred to growth medium containing 20% FBS. Total cellular RNA was isolated after serum stimulation for the intervals shown above each lane, and the level of c-*myc* mRNA was determined by Northern blot hybridization. The positions of *28S* and *18S* ribosomal RNA are indicated.

cytes that had been cultured in differentiating medium for 7 or 15 d, respectively, c-*myc* also was induced within 2 h in cardiac cultures that had lost one or both growth responses (cf. 9, 33). However, the expression of c-*myc* was not identical in older cells. With increasing duration of mitogen withdrawal, basal levels of c-*myc* mRNA estimated by scanning densitometry decreased to 0.5 of control, and the level of induction (compared with quiescent cells) diminished partially from 4.25-fold to 3.5-fold at 15 d. In addition, the kinetics of *myc* induction differed in ventricular myocytes maintained in culture for 15 d; maximal expression of c-*myc* was delayed (maximal at 24 h of serum stimulation) and was sustained.

To address the possibility that c-*myc* might be confined to a residual fraction of contaminating mesenchymal cells, the induction of c-*myc* mRNA was determined separately in myocyte and mesenchymal cell cultures that had been incubated for 7 d under identical conditions of mitogen withdrawal (Fig. 5). Serum induced c-*myc* mRNA to comparable levels (per microgram of RNA) in cardiac myocyte and mesenchymal cell cultures. For reference, the cultures also were stimulated with the α_1-adrenergic agonist, norepinephrine; in agreement with previous results (48), norepinephrine induced c-*myc* mRNA in ventricular myocytes, whereas cardiac fibroblasts did not respond. Since myocyte-enriched and myocyte-depleted cultures that were stimulated with serum contained levels of c-*myc* mRNA similar to one another, c-*myc* mRNA in myocyte cultures must largely be expressed in the dominant population of cardiac myocytes and not merely in the relatively few fibroblastic cells.

Serum Regulates α-Actin Gene Expression in Ventricular Muscle Cells Even after the Loss of Growth Responses

To obtain independent evidence for the persistence of serum effects on gene expression at each of the phenotypic stages identified above, we investigated regulation of the α-cardiac

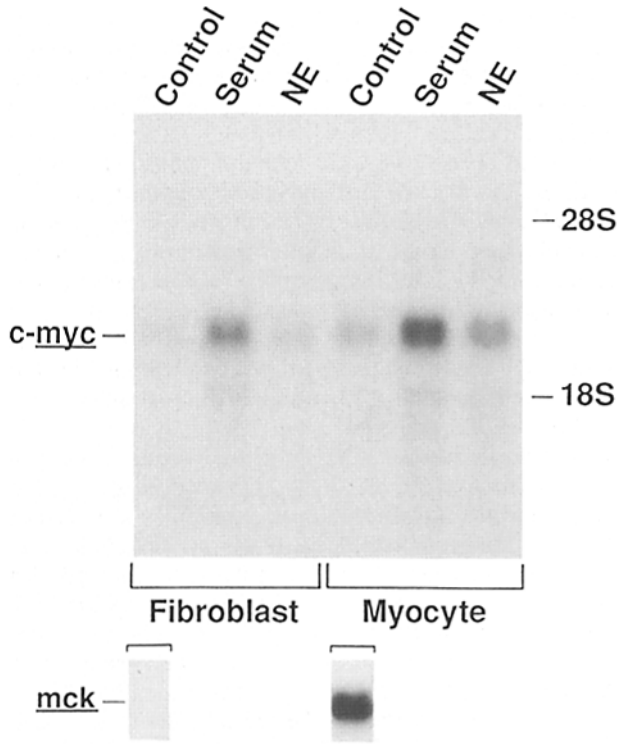

Figure 5. Serum induction of c-*myc* mRNA in ventricular myocyte cultures cannot be accounted for by cardiac mesenchymal cells alone. Myocyte-enriched and myocyte-depleted ("fibroblast") cultures prepared as described in Materials and Methods were maintained in differentiation medium for 7 d and were then treated with growth medium containing 20% FBS for 2 h (serum) or with differentiation medium containing 10 μM norepinephrine for 1 h (*NE*). Total cellular RNA was isolated, and expression of c-*myc* was measured by Northern blot hybridization. The positions of *28S* and *18S* ribosomal RNA are shown. The relative abundance of *mck* mRNA in myocyte-enriched and fibroblast cultures also is shown.

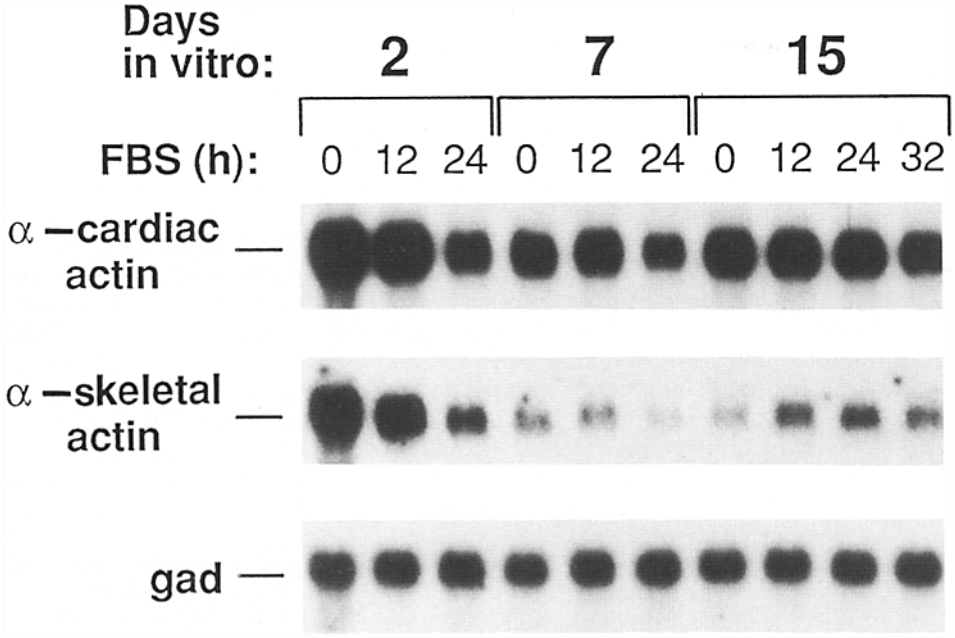

Figure 6. Expression of α-cardiac and α-skeletal actin mRNA in ventricular myocytes during differentiation elicited by mitogen withdrawal and during serum stimulation. The cultures were maintained in differentiation medium for 2, 7, or 15 d and were then transferred to growth medium containing 20% FBS. The levels of α-cardiac and α-skeletal actin mRNAs were determined by Northern blot hybridization to ^{32}P-labeled synthetic oligonucleotide probes, as described in Materials and Methods and the legend to Fig. 2.

and α-skeletal actin genes, since analysis of the embryonic/neonatal isoform is not confounded by its presence in fibroblastic cells (like *bck*) or by fluctuations in thyroid hormone level (like β-MHC). Adult (α-cardiac) actin mRNA already was expressed in cultured ventricular myocytes after 2 d in differentiating medium, and continued to be expressed at similar levels for at least 2 wk without evidence of dedifferentiation (Fig. 6). By contrast, α-skeletal actin mRNA coexisted with α-cardiac actin mRNA after 2 d in differentiation medium but subsequently declined by at least 90%, corresponding to actin expression in the normal postnatal heart. The abundance of *gad* mRNA, which does not fluctuate during cardiac or skeletal muscle differentiation (32, 39) and is used to detect inconsistent mRNA loading and transfer, did not vary. Cardiac mesenchymal cells expressed neither α-actin mRNA species (data not shown).

To determine whether α-actin mRNA expression could be modulated by serum factors in ventricular muscle cells that no longer proliferate in the presence of serum, the abundance of α-cardiac and α-skeletal actin mRNA was analyzed after a mitogenic challenge, after differentiation for 2, 7, or 15 d (Fig. 6). 24 h after exposure to 20% FBS, the level of both α-cardiac and α-skeletal actin mRNAs was significantly decreased in ventricular myocytes that had been subjected to mitogen withdrawal for 2 or 7 d. Compared to mRNA abundance before adding serum, α-skeletal actin levels decreased to 0.48 and 0.71 of control, at 2 and 7 d, respectively. Corresponding values for α-skeletal actin were 0.27 and 0.22. Contrasting results were obtained in ventricular myocytes that had been maintained for 15 d in differentiation medium and lost the ability to replicate DNA. Serum reduced the abundance of α-cardiac actin mRNA to 0.76 of control at 24 h, but produced at least a 2.4-fold increase in levels of the α-skeletal actin transcript (Fig. 6). Fig. 7 summarizes the results of scanning densitometry for serum modulation of α-cardiac and α-skeletal actin mRNA, together with c-*myc*, during each of the three phenotypic states.

Discussion

Several lines of inquiry indicate that the regulation of proliferative growth and differentiation in cardiac myocytes may possess unique biological features whose molecular basis is largely unexplored. However, successful investigation of atrial or ventricular cardiac muscle cells has been compli-

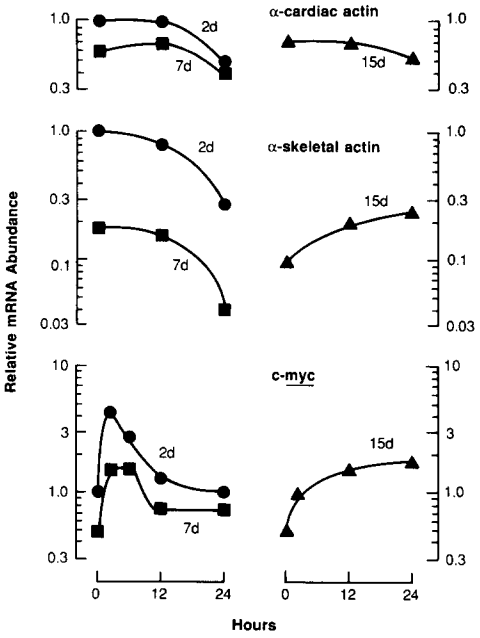

Figure 7. Mitogen withdrawal alters responses to mitogen challenge in cultured cardiac myocytes. The figure summarizes the results of Northern blot hybridization shown in Figs. 4 and 6, as analyzed by scanning densitometry. For each species of mRNA assayed, all values are normalized to the abundance observed in 2-d cultures at 0 h.

cated in conventional media not only by cellular heterogeneity but perhaps more importantly by inappropriate expression of embryonic protein isoforms. Although the use of mitogen withdrawal to promote the purity of cardiac muscle cells in culture is known (5, 28, 45), relatively little information has been available to establish whether mitogen withdrawal might also be sufficient to ensure the differentiated phenotype in cultured ventricular muscle cells. Noteworthy features of the in vitro preparation reported here include: (*a*) quiescence of the ventricular myocytes in the absence of exogenous growth factors; (*b*) spontaneous contraction maintained for 2 wk; (*c*) assembly of striations from day 6 onward; (*d*) a progressive increase in the fraction of muscle-specific subunits of CK; (*e*) stable expression of α-cardiac actin and MHC mRNAs for at least 15 d; and (*f*) appropriate down-regulation of the α-skeletal actin transcript. Thus, mitogen withdrawal was sufficient to ensure the developmentally accurate expression of cardiac-specific genes for at least 15 d and prevented the de-differentiation of ventricular myocytes which has been associated with cell culture in the presence of high concentrations of serum (cf. 28, 50, 52). Since abundance of α-skeletal actin mRNA normally is down-regulated between 15 and 30 d of age (25), the findings suggest that maturation of ventricular myocytes not only was maintained, but also progressed appropriately during the period of mitogen withdrawal. Simpson and co-workers identified the cross-striated morphology as an important indicator of differentiated structure, and regarded formation of striations in cultured ventricular muscle cells as representing a return to the original state of differentiation that had existed in vivo (44). Our results are consistent with the additional implication that mitogen withdrawal can enable the progression of developmentally regulated properties beyond the state found in ventricular myocytes at the time of their isolation.

Three sequential phases of growth control were identified in the model during progressively increasing intervals of mitogen withdrawal. These correspond to the three characteristic phases of growth observed in the perinatal rat heart in vivo (6, 30), indicated by the uncoupling of DNA synthesis from mitotic division as found in neonatal ventricular muscle. Little or no mitotic division and DNA replication are found in ventricular myocytes older than 3 wk of age, even under conditions of hemodynamic load that trigger hypertrophic growth (reviewed in reference 27). As previously suggested in chick cardiac myocytes (4), diminished growth responsiveness in rat cardiac muscle cells more closely resembled irreversible withdrawal from the cell cycle in skeletal muscle (29) than senescence in fibroblastic cells, whose decreased growth capacity involves, instead, increased duration of the G1 phase of the cell cycle (33). Taken together, the findings indicate that developmentally regulated properties of cardiac muscle can be accurately expressed in vitro, and the culture system characterized here functions as a suitable model with which to investigate the underlying molecular processes that control cardiac growth and differentiation.

The hypothesis that expression of the cellular oncogene c-*myc* might be linked to the control of growth in ventricular muscle cells was suggested by several independent forms of evidence cited above. Our results contrast with the simple correlation between activation of c-*myc* and either DNA synthesis or cell proliferation, and should be viewed in the context of an increasing number of observations that c-*myc* expression by itself is neither sufficient nor necessary to evoke the phenotypic changes prompted by various growth signals (reviewed in references 38, 40). Our findings described here extend the recent report that serum mitogens can provoke c-*myc* expression even in L6E9-B skeletal muscle cells that have undergone terminal differentiation (9). Since postmitotic myotubes also fail to respond in most respects to transforming growth factor β, the most potent known regulator of muscle differentiation, despite the presence of functional receptors for the peptide (23), the failure of c-*myc* induction to affect terminally differentiated skeletal muscle cells cannot necessarily be extrapolated to other myogenic systems. However, the kinetics of c-*myc* expression were rapid and transient in both L6E9-B myoblasts and myotubes (and in cardiac myocytes cultured for 2 d) whereas, after mitogen withdrawal for 15 d, up-regulation of c-*myc* in ventricular myocytes was delayed and sustained. Delayed accumulation of c-*myc* also has been observed during growth mediated by autocrine factors (21).

The results of our experiments also show that ventricular muscle cells that do not replicate DNA can respond to serum with induction of α-skeletal actin mRNA. This finding is consistent with the known ability of serum to provoke other aspects of hypertrophy, such as increased cell volume and protein (44). Although α-skeletal actin is abundant in the embryonic or newborn heart, and α-cardiac actin is found, analogously, in immature skeletal muscle, the relationship between these two genes is not reciprocal in most other respects. For example, primer extension studies have shown that equivalent levels of the two transcripts coexist in early embryonic limb and cardiac muscle (31), and in situ hybridization has recently demonstrated that induction of α-cardiac actin precedes α-skeletal actin expression in both lineages (36). Furthermore, numerous states and interventions exist that regulate both actin genes in tandem. In skeletal muscle, both α-actin genes are transcriptionally inactive during proliferation (1, 29), are induced at low levels in confluent cells even before serum withdrawal (1), are blocked by transforming growth factor β (23) or an activated c-H-*ras* gene (32), and are down-regulated by metalloendoprotease inhibitors (10) even in myotubes (15). In this context, suppression of both α-actin genes by serum in ventricular myocytes at 2 d of mitogen withdrawal resembles the coordinate inhibition of both sarcomeric actins in differentiated skeletal myocytes that have not undergone the transition to terminal differentiation (29).

Contrasting conditions also have been demonstrated that regulate α-skeletal and α-cardiac actin independently. Medium containing <150 μM Ca^{2+} specifically blocks α-skeletal actin induction in cultured chick myocytes (15). Conversely, L6E9 myoblasts fail to express α-cardiac actin, despite appropriate induction of α-skeletal actin (16). In rat cardiac muscle, aortic constriction and norepinephrine each provoke ~10-fold induction of α-skeletal actin mRNA, with no effect on the α-cardiac actin gene or modest stimulation (2, 14, 18, 41). Thus, although the relationship seen here between loss of growth capacity and control of a tissue-specific gene is distinct from terminal differentiation observed in skeletal muscle, the results in vitro are consistent with what is known to occur in adult cardiac muscle subjected to physiological growth signals. Hypertrophic growth and altered actin gene expression prompted by aortic constriction or norepineph-

rine also are preceded by induction of c-*myc* (19, 27, 48). Despite intriguing similarities between the responses of cardiac muscle to a hemodynamic load in vivo vs. adrenergic stimulation or serum mitogens in vitro, it is unknown whether these contrasting growth signals are coupled to cardiac mass and embryonic gene expression through related transduction pathways.

Serum contains a number of molecules other than peptide growth factors such as catecholamines which might, in principle, account for a portion of the results described here. However, the concentration of norepinephrine in medium supplemented with 20% FBS (~60 pM; reference 45) is three orders of magnitude below the minimal concentration required to activate α-skeletal actin mRNA (2). Furthermore, thyroid hormone effects the expression of myosin heavy chain genes, but does not induce a transition from α-cardiac to α-skeletal actin (14). Serum is a potent but crude growth agonist that exerts striking, bifunctional effects on α-skeletal actin mRNA (inhibition at a stage when it is normally expressed; induction at a stage when it is normally silent). The ability of single growth factors to exert multifunctional effects, depending on the state of differentiation, has been recently reviewed (47). While the issue of identifying the exact serum molecules that regulate cardiac myocyte growth and gene expression cannot be resolved by the data presented here; this in vitro model provides a basis for such studies. Although the potential involvement of innervation, cell work, and constituents of the extracellular matrix have not yet been resolved, it seems likely that mitogen withdrawal, by itself, can overcome certain of the obstacles that have precluded a molecular dissection of cardiac myogenesis in vitro.

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