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Dissociated Expression of c-*myc* and a *fos*-Related Competence Gene During Cardiac Myogenesis

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Cardiac myocytes irreversibly lose their proliferative capacity soon after birth, and cardiac DNA synthesis becomes uncoupled from mitotic division. Therefore, we examined cardiac muscle for developmental down regulation of inducible proto-oncogenes associated with cell proliferation. c-*myc* mRNA decreased continuously from day 13 of embryonic development and was dissociated from expression of the *fos*-related gene r-*fos*, which decreased precipitously between days 3 and 7 after birth.

In cardiac muscle, recovery from infarction or other injury is thwarted by the virtually irreversible loss of the ability of cardiac myocytes to divide soon after birth (33, 44). DNA synthesis in ventricular myocardium is subsequently dissociated from mitotic division, resulting in the formation of polyploid cardiac myocytes (14, 37, 38). Despite the implications of recent evidence that cellular proto-oncogenes may have a critical role governing cell proliferation (4, 16, 22), little or nothing is known of their expression in cardiac muscle. In particular, autonomous *myc* expression (1, 36) or microinjected *myc* protein (18) can in some circumstances abrogate the requirement for specific growth factors. Therefore, we examined the myocardial abundance of c-*myc* mRNA, along with other cellular proto-oncogenes, during normal cardiac development.

From the earliest age examined (day 13 of embryonic development) through 16 weeks of age, the levels of the 2.4-kilobase c-*myc* mRNA in rat ventricular myocardium decreased continuously, as determined by Northern blot hybridization (13, 26; Fig. 1A). c-*myc* mRNA was most abundant at 13 days in utero and decreased to less than one-third of its starting value by 1 week after birth. Glyceraldehyde 3-phosphate dehydrogenase (*gad*) mRNA, expressed constitutively in other systems, did not vary significantly (Fig. 1B), thus excluding systematic fluctuations in the proportion of mRNA in the total cellular RNA. For comparison, muscle creatine kinase (*mck*) mRNA levels were determined as an indicator of myocardial differentiation (35; Fig. 1C). In contrast to c-*myc*, *mck* mRNA accumulated monotonically and then plateaued at 4 weeks, as observed for α-myosin heavy-chain mRNA (25). Thus, c-*myc* mRNA was relatively abundant in embryonic and perinatal cardiac muscle, which is able to replicate DNA and undergo mitosis. Conversely, in adult myocardium which has undergone irreversible withdrawal from the cell cycle, levels of c-*myc* mRNA were low. The time course was consistent with the decreasing fraction of cardiac myocytes that incorporate [^{3}H]thymidine during the late embryonic and perinatal period (37).

A c-*fos* cDNA probe revealed no signal at the anticipated 2.2-kilobase position of c-*fos* transcripts (Fig. 1D; 6, 7, 28, 30); the abundance of c-*fos* mRNA in certain other systems is below the threshold for detection by Northern hybridization. However, an embryonic transcript was detected at 1.8 kilobases, as previously described for the platelet-derived growth factor-inducible *fos*-related gene r-*fos* (7), homologous with sequences in the c-*fos* fragment used here (28). The results with an authentic r-*fos* probe supported the identification of this *fos*-related transcript as r-*fos* (Fig. 1E). r-*fos* mRNA levels did not decrease in utero but declined precipitously between days 3 and 7 after birth. Thus, the expression of r-*fos* transcripts was developmentally regulated in cardiac muscle, with expression related to but distinct from the temporal expression of c-*myc*. This acute perinatal time course for the down regulation of r-*fos* mRNA in rat myocardium is compatible with the loss of mitotic division in ventricular muscle at day 4, whereas cardiac DNA replication persists at least through days 6 to 14 (37). In contrast, there was no down regulation of c-Ha-*ras* (Fig. 1F), whose expression in model systems does not appear to vary with competence either to replicate DNA or divide (30).

Cultures of cardiac ventricular cells were purified for presumptive cardiac myocytes, depleted of fibroblasts by mitogen starvation (Fig. 2A through C), and then mitogen stimulated (with 20% fetal bovine serum [FBS]). Essentially all cardiac cells were quiescent initially and reinitiated DNA synthesis by 18 h (unpublished data), as determined by flow microfluorimetry after ethidium bromide staining (43). *myc* mRNA abundance had increased eightfold at 2 h and had returned to the base-line level by 24 h (Fig. 2D), as in other lineages (20, 30). In contrast, *mck* mRNA exhibited little down regulation at 2 h but decreased to less than 1/10 of its starting value by 24 h (Fig. 2D). It remains to be determined whether c-*myc* can be induced, or whether *mck* can be down regulated, in cardiac myocytes that cannot be induced to reinitiate DNA synthesis (older myocytes and, perhaps, long-term cultures.)

Our results are consistent with the hypothesis that the probability that a cardiac myoblast will exit the cell cycle, as opposed to maintaining competence to proliferate, may be set by the level of c-*myc* and r-*fos* proteins or, implicitly, by their net effect in concert with differentiation-promoting factors. For myogenic cell lines, whether differentiation or continued division occurs depends in part upon ambient growth factor levels in the culture medium (24, 31, 34, 41; cf. references 12, 23, 32). Mutational analysis has suggested that quiescence, but not irreversible withdrawal from the cell cycle, may be obligatory for the induction of muscle-specific genes (40). However, cardiac muscle differs from skeletal

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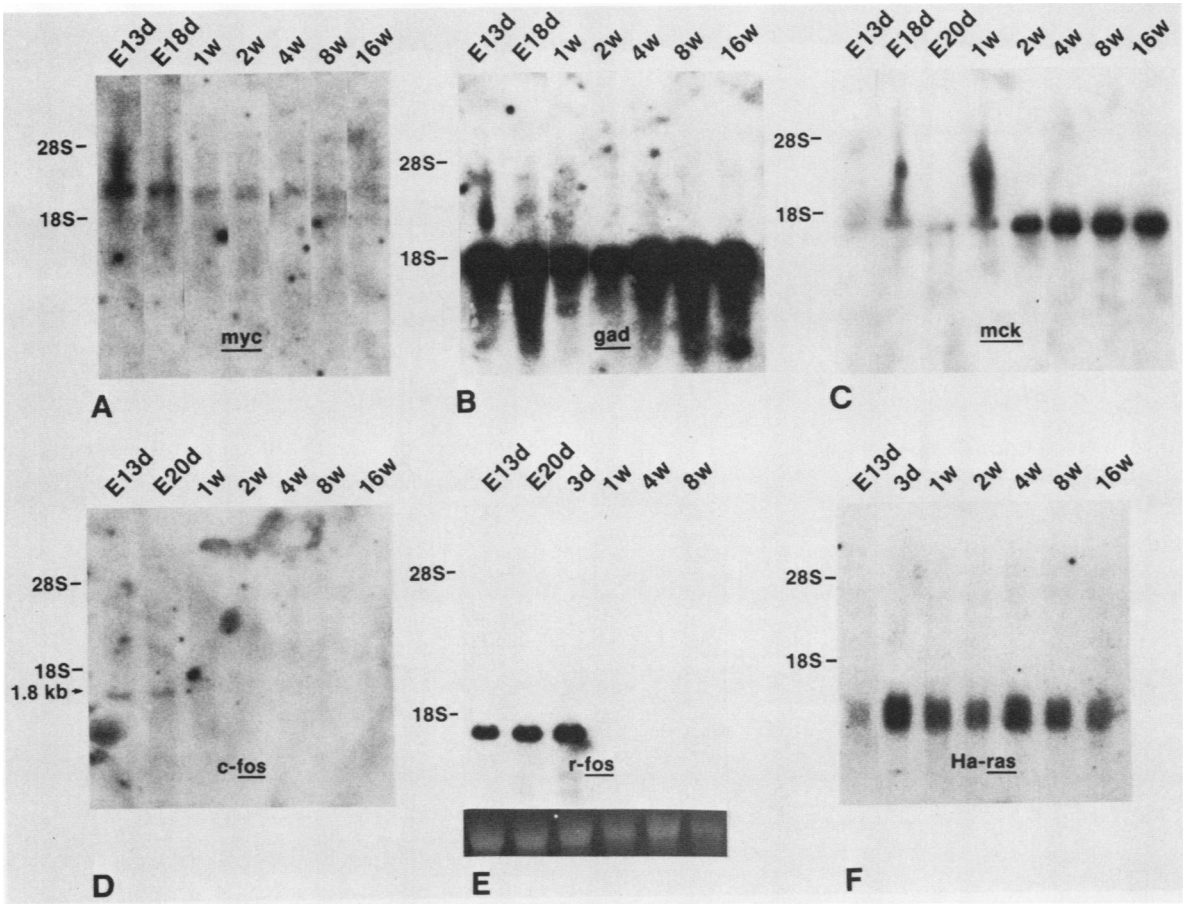

FIG. 1. (A through C) Accompaniment of cardiac differentiation by embryonic and postnatal down regulation of c-*myc* mRNA. Northern hybridization analysis used the c-*myc* (panel A), *gad* (panel B), and *mck* (panel C) cDNA probes specified below. (D and E) Precipitous decrease of *fos*-related transcripts between days 3 and 7 in cardiac muscle. Presumptive r-*fos* mRNA was detected at 1.8 kilobases (arrow) with the c-*fos* or r-*fos* hybridization probe. Parallel samples of stained 28S rRNA are shown below the autoradiogram in panel E. (F) Cardiac differentiation does not require down regulation of c-Ha-*ras* mRNA. The subgenomic fragments used as hybridization probes were as follows: *myc*, the 2.5-kilobase pair *Xba*I-*Hin*dIII fragment of pSVc-*myc*-1, comprising exons 2 and 3 of murine c-*myc* (22); *gad*, a 1.65-kilobase-pair *Hha*I fragment of pGAD-28 (10); *mck*, the 0.85-kilobase-pair *Pst*I fragment of pHMCK-Ia (M. B. Perryman, S. A. Kerner, T. J. Bohlmeyer, and R. Roberts, submitted for publication); *fos*, the 2.0-kilobase-pair *Eco*RI-*Sal*I fragment of pc-*fos*-3, comprising exons 2 through 4 of the murine c-*fos* gene (28); r-*fos*, the 1.05-kilobase-pair *Pst*I fragment of pBC-JB (7); and Ha-*ras*, the 0.8-kilobase-pair *Sma*I-*Pst*I fragment of Harvey murine sarcoma virus clone HB-11 (11). E, Embryonic; d, days; w, weeks.

muscle in that cell proliferation and induction of the differentiated phenotype are not simply reciprocal and mutually exclusive (19, 27). The low levels of *mck* mRNA present in cardiac muscle even at day 13 of embryonic development (Fig. 1C) support this observation and are in agreement with results of analogous reports of early induction of α-myosin heavy-chain mRNA (25).

Mechanisms that control c-*myc* mRNA abundance in the developing myocardium might plausibly include diminished proportions of one or more high-*myc* subpopulations, transcriptional control, a block to elongation, or mRNA transport, processing, or stability (3, 5, 20). Since mRNA levels are relatively invariant as a cell traverses the cell cycle during logarithmic growth (42), our findings indicate that gradations of cardiac *myc* mRNA abundance represent the declining proportion of cells that remain competent to replicate DNA and divide (37, 45). It is important to test this prediction by in situ hybridization with exon 1 and exon 2 probes (3). c-*myc* and r-*fos* mRNA levels in ventricular myocardium may in turn reflect alterations of exogenous or autocrine growth factors (29, 39) or age-related changes in transduction of mitogenic signals (2, 23). Therefore, it will be of interest to determine inducible levels of c-*myc* mRNA in cardiac muscle subjected to hemodynamic stresses that induce DNA replication and reexpression of fetal contractile proteins (17). Dissociated expression of c-*myc* and r-*fos* during the transition from hyperplastic to hypertrophic growth may also have implications for molecular mechanisms that uncouple DNA synthesis from mitotic division in the myocardium.

Our observations should be interpreted cautiously, however, in view of recent investigations that failed to confirm either a direct relationship between c-*myc* induction and DNA synthesis or, alternatively, between c-*myc* down regulation and the induction or maintenance of the differentiated state (6, 9, 12). Therefore, it is critical to test more directly the possible functional role for c-*myc* and other putative regulatory elements that might influence cell proliferation or differentiation, by gene transfer, to uncouple *myc* expression from exogenous growth factors in a model myogenic system. Analogously, constitutive expression of c-*myc* driven by a viral promoter has been shown to block

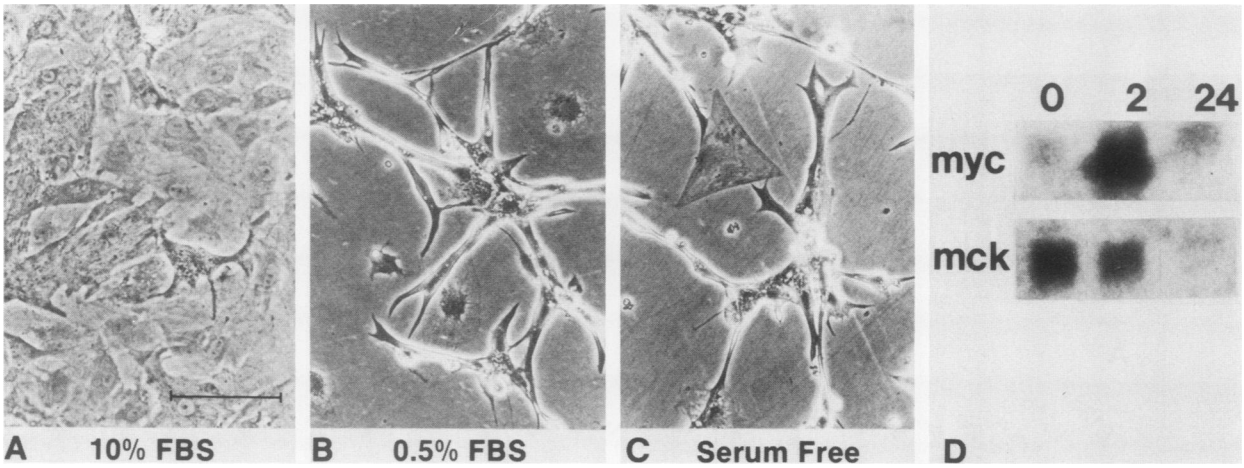

FIG. 2. (A through C) Phase-contrast microscopy of rat ventricular myocardial cells cultured in reduced FBS or serum-free medium for 7 days. The constituents of the media were as follows: panel A, Dulbecco modified Eagle medium-Ham F12 medium (1:1), 17 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4), 3 mM $NaHCO_3$, 2 mM L-glutamine, 50 μg of gentamicin per ml, and 20% heat-inactivated FBS; panel B, same as panel A except FBS reduced to 0.5%; panel C, serum-free medium modified from that of Hayashi and Kobylecki (15) and containing 5 μg of insulin per ml, 5 μg of transferrin per ml, 10 μM hydrocortisone, 1 ng of angiotensin II per ml, 1 nM Na_2SeO_4, and 1 nM LiCl. Bar = 100 μm. (D) Northern hybridization analysis of c-*myc* and *mck* mRNA in quiescent cardiac myocytes treated with FBS. Myocardial cell cultures (day 18 of embryonic development) were induced to differentiate for 48 h in medium containing 0.5% FBS and were then treated with 20% FBS for the number of hours shown above each lane. Comparable results were obtained after 48 in the serum-free medium (not shown).

differentiation of mouse erythroleukemia cells (8). In contrast, our recent studies of myocytes transfected with a transcriptionally activated *myc* gene or a truncated *erbB* gene (M. D. Schneider, M. B. Perryman, P. A. Payne, G. Spizz, R. Roberts, and E. N. Olson, submitted for publication) indicate that autonomous *myc* expression by itself prevents neither withdrawal from the cell cycle nor induction of muscle differentiation products, whereas complementary oncogenes such as *myc* and *erbB* genes (cf. reference 21) can interact cooperatively to inhibit myogenic development.

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