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c-myc Gene Expression Is Localized to the Myocyte Following Hemodynamic Overload In Vivo

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Abstract Expression of the proto-oncogene c-myc increases in the hemodynamically overloaded heart, but expression by cardiac myocytes has not been shown. To address this issue, right ventricular overload was induced in cats by pulmonary artery banding. Expression of c-myc and α-skeletal actin mRNA were determined by Northern analysis. Immuno-reactive Myc protein was identified by histochemical staining.

Steady state levels of c-myc mRNA peaked within 2 h after banding. Levels of α-skeletal actin mRNA were maximally increased 48 h–1 week after banding and were still elevated at 1 month. Prominent staining of myocyte nuclei for immunoreactive Myc protein was detected 48 h after banding although a few interstitial nuclei were also positive.

These studies show that c-myc and α-skeletal actin gene expression are upregulated in a large animal model of hemodynamic overload. The localization of the immunoreactive Myc protein to right ventricular myocyte nuclei after pulmonary artery banding supports the hypothesis that c-myc induction is part of a general response in cardiac hypertrophy that is common to many mammalian species. © 1994 Wiley-Liss, Inc.

Key words: hypertrophy, proto-oncogene, c-myc, actin, pressure-overload, myocyte

Growth-regulated genes, among them the proto-oncogene c-*myc,* are induced in the early stages of experimental cardiac hypertrophy [Simpson, 1988; Parker and Schneider, 1991]. Steady-state levels of c-myc mRNA are elevated in the pressure-overloaded rat left ventricle after aortic banding [Mulvagh et al., 1987; Izumo et al., 1988; Komuro et al., 1988], but these studies, using RNA isolated from whole cell homogenates, did not identify the cell-type expressing c-myc. While c-myc gene is induced in hypertrophying neonatal rat myocytes in vitro [Starksen et al., 1986], it is not clear that results obtained with this in vitro model necessarily reflect the situation in vivo. The interstitium proliferates in cardiac hypertrophy [Morkin and Ashford, 1968] so, the induction of c-myc may in vivo be a marker for nonmyocyte proliferation. In fact, Snoeckx and co-workers found c-Myc immunoreactivity to be localized exclusively to the interstitium in rat hearts 12 h after induction of hemodynamic overload [Snoeckx et al., 1991]. Their results suggest the possibility that the increased levels of c-myc mRNA observed by others in the pressure-overloaded heart [Mulvagh et al., 1987; Izumo et al., 1988] are reflective of induction by the interstitial cells. It is also possible that alterations in cardiac gene expression occurring in response to a hypertrophic stimulus in one species may not necessarily occur in other species. While alterations in myosin gene expression occur in response to a pressure overload in the rat ventricle and are directly related to changes in cardiac contractile function, functionally significant myosin isoenzyme changes do not seem to occur in response to hemodynamic overload in the human ventricle [Swynghedauw, 1988].

Given these observations, the present study was performed in a well characterized feline model of right ventricular pressure overload [Spann et al., 1967] in order (1) to establish that c-myc gene expression is part of a generalized myocardial response in pressure-overload cardiac hypertrophy, (2) to determine the cell-type responsible for its expression, and (3) to determine whether alterations in contractile protein α-skeletal actin gene expression occur in this model in pressure overload. Steady-state levels

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of α-skeletal actin mRNA increase in the pressure-overloaded rat heart [Schwartz et al., 1986; Izumo et al., 1988], but the response in larger mammals has not been well studied.

MATERIALS AND METHODS

Pulmonary Artery Banding

Cats of either sex were used in this study. Animals were anesthetized with ketamine (50 mg/kg) and acepromazine (0.5 mg/kg), intubated, and placed on a positive-pressure ventilator. Under sterile conditions, an incision was made in the left thorax one interspace superior to the cardiac apical impulse. The pericardium was entered and the pulmonary artery was dissected free of the ascending aorta and constricted with a band 3.2–3.5 mm in diameter depending on the weight of the animal [Spann et al., 1967]. Sham-operated animals had a band loosely placed around the pulmonary artery which was immediately removed. This protocol has the approval of Temple University's Institutional Animal Care and Use Committee. A total of 10 animals underwent pulmonary artery banding and 9 animals underwent the sham procedure.

Animals were sacrificed under pentobarbital sodium anesthesia (30 mg/kg) within 2 h, 48 h, 1 week, and 1 month after surgery. Right ventricular systolic pressures (RVSP) and right ventricular end diastolic pressures (RVEDP) were measured prior to sacrifice in the 48 h and 1 month groups. The chest was opened and the heart was rapidly excised and weighed. The right ventricular free wall was dissected free and snap frozen in liquid nitrogen. Tissue was stored at −70°C until the time of analysis.

Northern analysis was performed on individual samples of RNA isolated from the right ventricle of each animal. Immunohistochemical analysis was performed on frozen sections from most of these ventricles. Feline spleen was used as a positive control for Northern analysis of c-myc expression and for immunohistochemistry.

Northern Analysis

RNA was isolated from ventricular tissue by the guanidinium isothiocyanate method [Chomczynski and Sacchi, 1987]. Northern blotting was performed according to standard methods [Sanbrook et al., 1989]. Total RNA (20–40 μg) was separated on 2.2 M formaldehyde/1.2% agarose gels and transferred to nitrocellulose filters. Prehybridizations and hybridizations were done at 42°C in 50% formamide. The probes used in this study were the c-myc insert from the plasmid pSVCmyc-1 [Land et al., 1983] and pHMαA-3′ut which contains the 3′ isoform specific untranslated region of the human α-skeletal actin gene [Ponte et al., 1983]. Among the actins, there is a high degree of sequence homology within the 3′-untranslated regions of each particular actin isotype and its analog in other vertebrate species [Ponte et al., 1983]. Probes were labeled with ^{32}P deoxycytidine 5′-triphosphate (^{32}P dCTP) using the random primer method [Feinberg and Volgelstein, 1983]. Filters were washed 3 times for 5 min each in 2× SSC (150 mM NaCl, 15 mM Na Citrate, 0.1% SDS) at 23°C, then 3 times for 30 min each in 0.1× SSC at 52°C. Autoradiographs were exposed at −70°C for 18–24 h with intensifying screens and quantitated by scanning densitometry on a LKB Ultroscan XL Laser Densitometer.

Immunohistochemistry

Frozen sections of myocardial tissue were prepared and stained for Myc as described [Bading et al., 1989], using a commercially available affinity-purified sheep polyclonal antibody raised against a synthetic peptide sequence that is totally conserved in mouse and human c-Myc (OA-11-801, Cambridge Research Biochemicals, Valley Stream, NY). The antibody was used at a final concentration of 25 μg/ml. Incubation with antibody was followed by alkaline phosphatase detection with a Vectastain ABC-AP Kit (Vector Laboratories, Burlingame, CA). Nonimmune sheep serum was used for staining controls. Sections from banded and sham-operated animals were always analyzed simultaneously under the same conditions using the same reagents.

Values for body weights are reported as means ± standard deviations (SD). Statistical differences between mean values for two groups were evaluated by the unpaired Students' *t*-test with a P value of ≤ 0.05 considered significant.

RESULTS

Physiological Characteristics.

Body weights (kg) at the time of sacrifice were similar in banded (n = 10) and sham-operated (n = 9) animals (2.5 ± 0.46 versus 2.4 ± 0.51; P = NS). The right atrium and ventricle were dilated in those banded animals sacrificed within

48 h after surgery. Right ventricles in 1-month banded animals were grossly thickened as compared with 1-month sham-operated controls consistent with the development of right ventricular hypertrophy. Heart weights in the 1-month banded animals were increased (14 and 19 g) vs. the control hearts (9 and 12.5 g). Heart weight/body weight (g/kg) ratios were also increased (5.3 vs. 3.8).

Hemodynamic measurements were obtained in animals sacrificed at the 48 h and 1-month time points. Pulmonary artery banding resulted in sustained elevations of right ventricular pressures (Table I). Right ventricular systolic and diastolic pressures were elevated 48 h after pulmonary artery banding as would be expected with the induction of an acute hemodynamic overload. Right ventricular systolic but not diastolic pressures remained increased 1 month after surgery, consistent with the development of compensated right ventricular hypertrophy.

Northern Blot Analysis of c-myc Gene Expression

Figure 1 shows the results of Northern analysis of c-myc gene expression in banded and sham-operated ventricles. Northern analysis was performed on RNA isolated from individual hearts. The results shown are representative of findings obtained from 2 or 3 individual hearts at each time point. An intense c-myc signal was detected within 2 h after pulmonary artery banding. Only a very weak signal was seen in RNA from sham-operated ventricles at the same time point. Steady-state levels of c-myc mRNA were still somewhat elevated 48 h after banding but returned to baseline 1 week after surgery.

TABLE I. Hemodynamic Characteristics of Pulmonary Artery and Sham-Banded Animals*

Time	Condition	Heart rate (bpm)	RVSP (mm Hg)	RVEDP (mm Hg)
48 h	Banded[a]	195	40	12
	Banded	180	34	9
	Sham	240	20	0
	Sham	180	28	0
1 mo	Banded	160	54	4
	Banded	195	40	6
	Sham	180	24	6
	Sham	180	24	6

*Values are as measured in individual animals at the time of sacrifice.
[a]Two of 3 animals. The catheter could not be passed into the right ventricle in one cat.

Northern Blot Analysis of α-Skeletal Actin Gene Expression

α-skeletal actin mRNA was present in RNA isolated from both banded and sham-operated ventricles (Fig. 2). While steady-state levels were elevated in the banded ventricles relative to controls at all time points, this increase was maximal 48 h–1 week after surgery.

Figure 3A shows that the time courses of maximal c-myc and α-skeletal actin gene expression differed in banded animals. In contrast, the relative expression of c-myc to α-skeletal actin was fairly constant for the sham-operated ventricles (Fig. 3B).

Immunohistochemistry

Numerous myocyte nuclei were positive for immuno-reactive Myc 48 h after pulmonary artery banding (Fig. 4A). Sections from a 48-h sham-operated ventricle (processed simultaneously under the same conditions and with the same reagents as the banded ventricle shown in Fig. 4A) were entirely negative for immuno-reactive Myc (Fig. 4B). No staining could be detected in control sections that were incubated with nonimmune sheep serum (not shown).

Immuno-reactivity to c-Myc was maximal 48 h after pulmonary artery banding; still, a few positive myocyte nuclei could be detected as early as 2 h after pulmonary artery banding. Positive myocyte nuclei persisted for one week after pulmonary artery banding but were never seen at 1 month. An occasional positive myocyte nucleus could be found in sections from sham-operated ventricles 2 h after surgery; sections from sham-operated animals sacrificed at later time points were consistently negative.

DISCUSSION

The results of this study support the hypothesis that c-myc induction is part of a generalized response to a hemodynamic stimulus in the mammalian heart. That c-myc is expressed early following pressure-overload has already been shown [Mulvagh et al., 1987; Izumo et al., 1988; Komuro et al., 1988], and in this regard, our study confirms results obtained by these investigators. However, this study expands those findings in several ways. Firstly, c-myc induction is part of a generalized mammalian response to hemodynamic overload since it occurs in the hearts of other species besides rodents. Secondly, the myocyte is the predominant cell-type

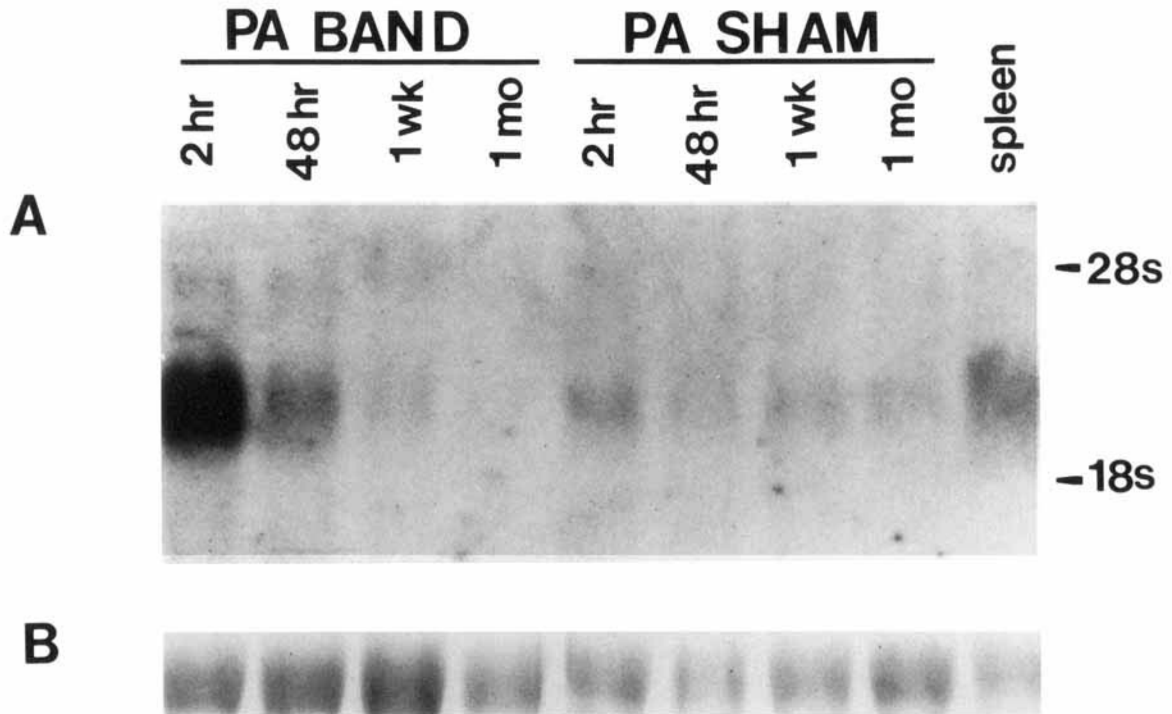

Fig. 1. **A:** Northern blot of c-myc gene expression in pressure-overloaded feline right ventricles following pulmonary artery banding. Time points are as indicated. RNA isolated from feline spleen was used as a control. **B:** Ethidium bromide staining of the 28S ribosomal RNA subunit.

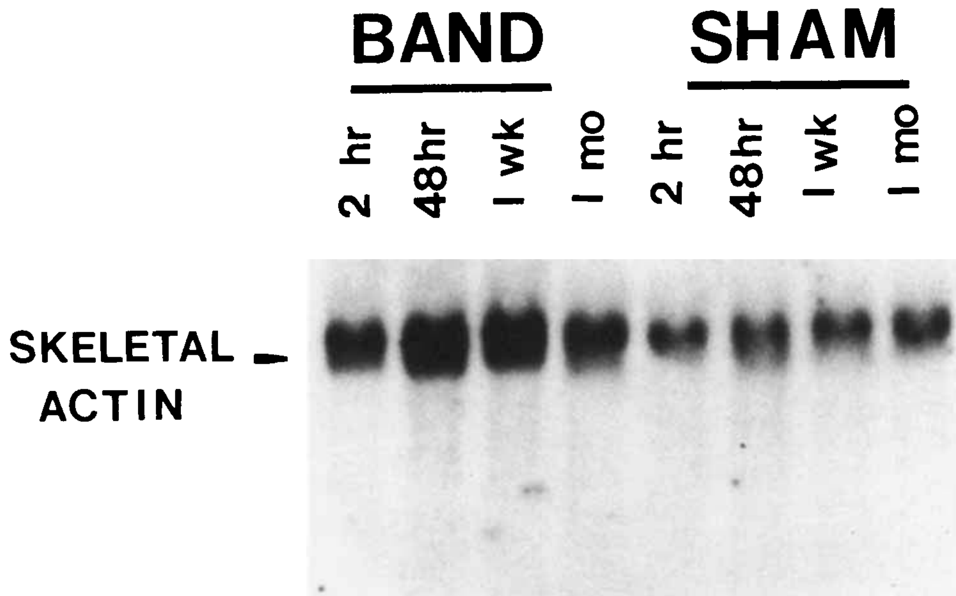

Fig. 2. Same blot as in Figure 1, stripped and reprobed with PHMαA-3′ut.

expressing c-myc in pressure overloaded myocardium. The detection of immunoreactive Myc within myocyte nuclei argues that this is a response of biological significance.

The time course for the appearance of Myc immunoreactivity in cardiac myocytes following the imposition of a hemodynamic load in vivo parallels the time course previously observed by

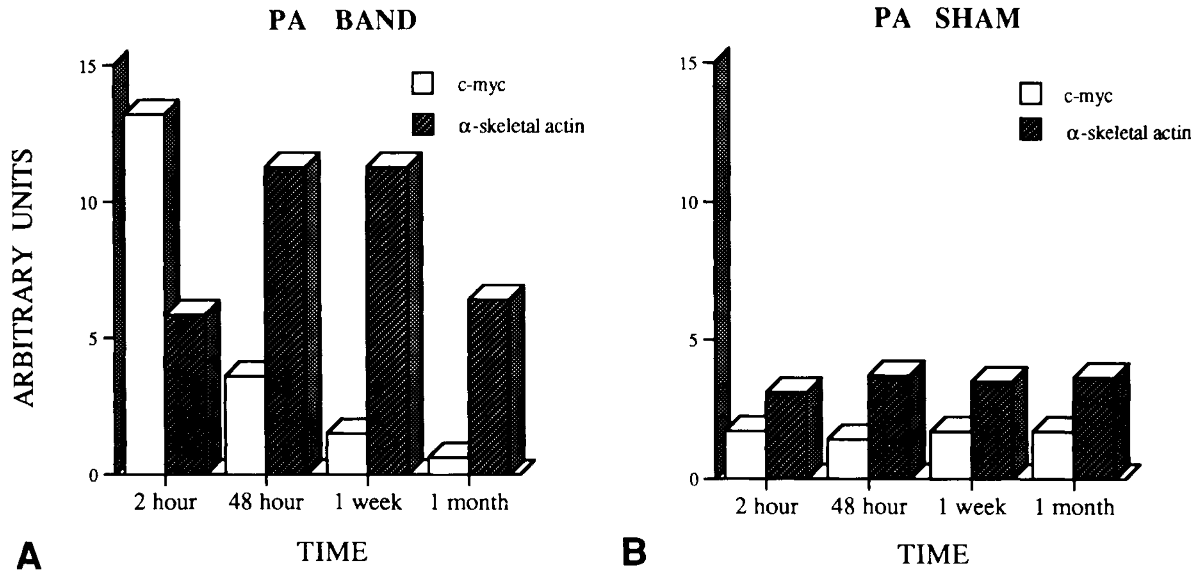

Fig. 3. Steady-state levels of c-myc and α-skeletal actin mRNAs versus time after surgery. The blots shown in Figures 1 and 2 were scanned by laser densitometry and the relative areas under the curve are expressed in arbitrary units. **A:** Pressure-overloaded right ventricle. **B:** Sham-operated right ventricle.

others in proliferating cells. For example, when quiescent NIH3T3 cells are reactivated by plating at low density in serum, the level of Myc protein reaches its maximal induced level 24–48 h after plating and then decreases rapidly [Rabbits et al., 1985]. This is in agreement with the hypothesis that certain aspects of cellular proliferation and myocardial hypertrophy may be regulated in similar fashion [Parker et al., 1991].

The role of c-myc in pressure-overloaded myocardium remains speculative. It is a transcriptional regulator [Blackwell et al., 1990; Prendergnast and Ziff, 1991] and the specific localization of Myc to the myocyte in the early stages of cardiac hypertrophy suggests that Myc may mediate in the upregulation of cardiac gene transcription that occurs in response to a hemodynamic stimulus. The proto-oncogenes c-fos and c-jun have recently been shown to directly regulate α-skeletal actin gene expression in neonatal rat heart cells [Bishopric et al., 1992] supporting the idea that proto-oncogenes regulate transcription in response to hypertrophic stimuli. Fos has been localized to myocyte nuclei in perfused rat hearts that acutely generate a high systolic wall stress [Schunkert et al., 1991]. The results of the current studies support the need for more detailed investigations into mechanisms by which c-myc may regulate the development of cardiac hypertrophy.

In these experiments, c-myc induction was rapid and paralleled the acute increase in right ventricular load, which is characteristic of this animal model. It is possible that the pattern of c-myc induction might be different if the hemodynamic overload were gradual and progressive. We have recently developed a feline model of gradual and progressive left ventricular overload which more closely mimics human disease in its ongoing nature. In this model, the degree of overload steadily increases with time and the extent of hypertrophy of individual myocytes is greater than in right ventricular overload [Bailey and Houser, 1992; Pollack et al., 1993]. Such a model will be useful in the examination of this question.

A small increase in c-myc expression was seen in sham-banded animals at the 2-h time point only. In the sham-banding procedure used in our laboratory, the pulmonary artery is dissected free and a band is placed around the vessel and immediately removed. The inevitable manipulation of the pulmonary artery during this process may have led to transient increases in right-sided pressures. This is the most likely explanation for the slight increases in steady-state levels of c-myc mRNA and the rare positive immunoreactive myocyte nucleus observed 2 h after sham operation. We have been unable to

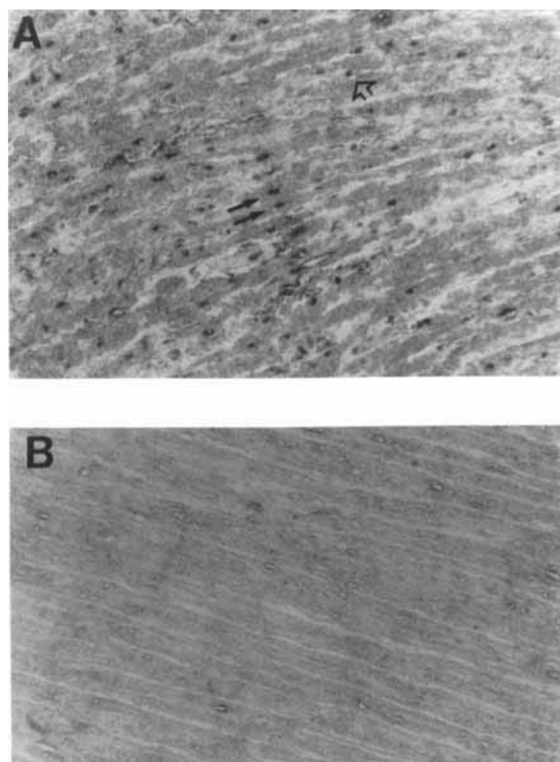

Fig. 4. Detection of immunoreactive Myc protein. A: Pressure-overloaded ventricle 48 h after pulmonary artery banding. Numerous myocyte nuclei are immunopositive (arrows). A positive interstitial nucleus is indicated by the arrowhead. Nuclear staining was present in multiple sections from this ventricle and similar findings were present in a duplicate experiment. B: Sham-operated ventricle 48 h after surgery. This section was processed simultaneously, under the same conditions and with the same reagents as the section shown in A. There is no nuclear staining. ×75.

detect c-myc mRNA in the myocardium of unoperated control cats (not shown).

Occasional interstitial cell nuclei were reactive for c-myc. This observation is entirely expected as considerable interstitital proliferation has long known to be characteristic of cardiac hypertrophy [Morkin and Ashford, 1968]. It is also consistent with the results obtained by Snoeckx and co-workers who used the same antibody as in our studies (a sheep polyclonal antibody raised against a c-Myc amino acid sequence that is totally conserved in the mouse and human [Snoeckx et al., 1991]). These workers found c-Myc immunoreactivity to be localized exclusively to coronary endothelium and myocardial nonmuscle cells 3 and 12 h after aortic banding; myocyte staining was not seen at these early time points and no later time points were studied. In our experiments, maximal c-Myc immunoreactivity was present 48 h after pulmonary artery banding and it was also at this time point that myocyte nuclei were most clearly reactive to the Myc antibody. Snoeckx's inability to detect nuclear localization of Myc in myocytes is most likely related to the early time points chosen for study by those investigators.

Steady-state levels of α-skeletal actin mRNA were increased in the feline ventricle 48 h after pulmonary artery banding and elevated levels persisted one month later. Skeletal actin mRNA levels are increased in pressure-overloaded rat hearts [Schwartz et al., 1986; Izumo et al., 1988]. Bakerman and co-workers have more recently shown that steady-state levels of α-skeletal actin mRNA increased in the right, but not the left ventricles of newborn calves exposed to hypobaric hypoxia. These charges were partially reversible when the calves are removed from the hypobaric chamber [Bakerman et al., 1990]. Since it is known that the expression of this gene is mediated at the transcriptional level in the isolated neonatal rat myocyte [Long et al., 1989], it would seem that the up-regulation of α-skeletal actin is a specific response to an increased load and is likely to be regulated, at least in part, at the transcriptional level. The time courses of c-myc and α-skeletal actin gene expression in this model are consistent with the hypothesis that Myc may play some role in mediating α-skeletal actin gene expression as has already been shown for Fos and c-Jun [Bioshopric et al., 1992]. However, the present experiments were not designed to demonstrate a cause and effect relationship in this regard and can only point to areas where further work is needed.

In conclusion, the results of these studies demonstrate that the expression of both c-myc and α-skeletal actin is upregulated in a large animal model of hemodynamic overload. The time courses of expression of these two genes are different. Furthermore, Myc can be localized to the myocyte by immunohistochemical techniques 48 h after hemodynamic overload is induced. The results of these experiments are consistent with the hypothesis that the *c-myc* gene product is a transcriptional mediator in the early stages hemodynamic overload. Further studies are needed to determine mechanisms by which Myc may regulate the myocyte's response to hemodynamic overload.

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