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Expression of proto-oncogenes and muscle specific genes during cardiac hypertrophy and development in rats and humans

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Abstract A regulatory interdependence of expression of proto-oncogenes and muscle specific genes observed in smooth muscle was examined in cardiac muscle during normal development and hypertrophy both in rats and humans. During normal development in rats, myosin light chain 2 expression is very low at prenatal stages, while c-fos expression starts from the early stages of embryonic development. In aorta constricted rats c-fos induction occurs within 30 min whereas myosin light chain 2 expression is sufficiently high only after 3 or 4 days of post operative period. In the case of humans, the expression of myosin light chain 2 as well as c-fos occurs at high levels during embryonic development. Similar results were obtained with tissue samples obtained from patients with cardiac abnormalities. Induction of the c-fos gene in cultured myocytes by 12-O-tetradeeanoylphorbol 13-acetate has no influence on the expression of myosin light chain 2. These studies were extended with studies on c-myc and β-myosin heavy chain gene expression which revealed a similar pattern of expression as that of c-fos and myosin light chain 2. These results have indicated that the expression of proto-oncogenes in cardiac muscle may be independently regulated from the expression of muscle specific genes.

Keywords. Atrial septal defect; cardiac hypertrophy; c-myc; mysoin heavy chain (MHC); myosin light chain 2; tetralogy of Fallot; TPA.

1. Introduction

Induction of cellular oncogenes has been proposed to regulate the transcription of several other genes. For example, the enhanced expression of c-fos gene which encodes a nuclear protein, acts as a "master switch" in controlling gene expression during differentiation and in response to external stimuli (Distel *et al* 1987). The activation of expression of proto-oncogenes such as c-myc and c-ras down regulates the expression of muscle specific genes (Olson *et al* 1987; Schneider *et al* 1987). It has been reported that, in c-ras transformed HOS cell lines, the smooth muscle myosin light chain 2 (MLC2) expression is specifically repressed (Kumar and Chang 1992). Similarly in skeletal mvogenesis, activation of c-fos gene expression is associated with the inhibition of several muscle specific genes (Lassar *et al* 1989) whereas in cardiomyocytes, the induction of c-fos and c-jun by adrenergic agonists

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is associated with the assembly of MLC2 (Iwaki *et al* 1990). This might be due to the positive regulation of c-fos on the specific regulatory factors which activate MLC2 gene expression. This is in contrast to the report of Goswami *et al* (1992) who have recently shown that, the expression of c-fos inhibits the transcriptional activity of chicken cardiac MLC2 promoter in cultured embryonic chicken cardiac muscle cells.

The present study was carried out to examine whether expression of proto-oncogenes has any effect on the expression of muscle specific genes in cardiac muscle during normal development and during myocardial hypertrophy. Studies with normally developed heart in both rat and human revealed that, the induced expression of proto-oncogenes occurs independent of muscle specific genes. Further, these studies showed that, the induction of proto-oncogenes during the development of cardiac hypertrophy does not have any effect on muscle specific gene expression. These studies suggest that these two classes of genes may be differentially regulated in cardiac muscle during normal development and myocardial hypertrophy in both humans as well as experimental animals (rats).

2. Materials and methods

2.1 *Tissue source*

To induce pressure overloaded hypertrophy of the hearts, albino rats of Wistar derived strains (120–130 g) were anesthetized with diethylether and the main ascending aorta constricted with a tantalum hemoclip (Edward Weck and Co. Cat. No. 523135) following the method of Rakusan and Poupa (1966) with minor modifications. Animals were killed at predetermined limes (0·5, 1,2,3,6, 12 and 24 h) after operation. Sham-operated animals were subjected to an identical procedure except for placement of the hemoclip. The development of hypertrophy was calculated as per cent increase in the ratio of heart weight (wet weight) to body weight with respect to sham-operated controls (Meenakshi *et al* 1983). To investigate the developmental changes, hearts of 10 and 20 days old embryos and 7 and 14 days old neonates were examined.

The 20 , 21, 2 3 and 28 week old human embryonic hearts were kind gift of Ms. Bonne Gisel, Paris, France. The atrial and ventricular biopsy tissues of cardiac patients with atrial septal defect (ASD) and tetralogy of Fallot were kind gift of Dr M S Valiathan, Director, Shree Chitra Thirunal Institute of Medical Sciences, Trivandrum. The autopsy samples for normal control were collected from healthy individuals who died due to road accident.

2.2 *Probes used*

cDNA fragments corresponding to the coding region of rat c-fos and c-myc genes were kind gifts from Prof. M A Q Siddiqui, Health Science Centre, Brooklyn, State University of New York, USA. Rat cardiac myosin heavy chain clone pcMHC5 is a kind gift of Prof. B Nadal Ginard. The 769 bp *Eco*RI fragment corresponding to the human cardiac MLC2 cDNA is of our own isolate.

2.3 *RNA isolation and Northern analysis*

Total cellular RNA was isolated by the guanidium thiocyanate method of Chomczynski and Sacchi (1987). For Northern blot analysis 20 μg of total RNA was size fractionated in a 1·2% formaldehyde/agarose gel and transferred onto nylon membrane (Ausubel *et al* 1987). Hybridization was carried out as described in "Membrane transfer and detection methods" Amersham (1985). Prehybridization was performed in a solution containing 6 × SSC, 50% formamide, 5 × Denhart's solution, 10% Dextran sulphate and 100 μg/ml heat denatured calf thymus DNA for 4 h at 42°C. Hybridization was carried out in the same solution with the addition of 10^5 cpm/ml ^{32}P-labelled probe for 18 h at 42°C. Probes were prepared using Amersham Nick translation kit. Membranes were washed twice at 42°C with 2 × SSC (saline sodium citrate) buffer and 0·1% SDS, twice at 42°C with 1 × SSC and 0·1% SDS, once at room temperature with 0·1 × SSC and 0·1% SDS, air dried and exposed to X-ray film for 48 h with an intensifying screen at – 70°C.

2.4 *Slot-blot analysis*

Different concentrations of total RNA were blotted onto the nylon membranes using BRL Hybri-slot blot apparatus. Hybridization was carried out as described earlier.

The autoradiograms were subjected to a densitometric scan using an LKB Ultrascan XL.

2.5 *Isolation of nuclei*

Nuclei were isolated and purified following the method of Mariappan *et al* (1990). The ventricular tissue was minced and homogenized in 8 vol. of an ice cold homogenizing buffer (0·3 M sucrose, 0·01 M Tris.cl (pH 7·5), 0·01 M NaCl, 0·001 M $MgCI_2$, 0·005 M $CaCI_2$ and 1 mM PMSF) using a motor driven Teflon homogenizer. The homogenate was filtered through four layers of cheese cloth and centrifuged at 1000 *g* for 10 min at 4°C. The crude nuclear pellet was purified by pelleting through a step gradient of 2·4 M and 1·6 M sucrose in TEN buffer [0·01 M Tris.cl (pH 7·5), 0·002 M EDTA and 0·01 M NaCI] at 90,000 *g* for 1 h at 4°C. The pellet was suspended in 1 M sucrose containing 0·001 M $CaCI_2$ and washed twice with the same buffer by centrifugation at 11,000 *g* for 10 min at 4°C.

2.6 *In vitro RNA synthesis*

In vitro transcription assay was carried out according to Long and Ordahl (1988) with slight modifications. The reaction was carried out at 25°C for 20 min in a 100 μl reaction volume containing 25 mM HEPES (pH 8) 1m M $MnCI_2$, 10 mM $MgCI_2$, 2·5 mM Mg(CH3COO)2, 10% (v/v) glycerol, 150 mM KCl, 12 mM BME, 0·2 mM spermidine, 2·0 mM creatine phosphate, 30 μg creatine phosphokinase, 600 μM each of ATP, GTP, and CTP, 60 μM UTP. 100 μCi [α-^{32}P]UTP (3000 Ci/mmol) and 2 × 10^5 nuclei. The reaction was stopped by adding 100 μl of 0·5% SDS-10 mM EDTA (pH 8) solution. RNA was extracted by phenol: chloroform (1:1) and ethanol precipitated.

2.7 *Isolation and culturing of cardiomyocvtes*

Cardiomyocytes from the ventricles of sham-operated and 28% hypertrophied rats were isolated following the method of Nair and Gupta (1988). Ventricular tissue was minced in a petridish containing balanced salt solution (BSS) and transferred into a trypsinization flask containing a teflon magnetic bar, 10 ml of dissociation medium containing 0·3 mg/ml collagenase, 0·3 mg/ml trypsin, 1 mg/ml bovine serum albumin in Hank's BSS and antibiotics (Penicillin 100 U/ml, gentamycin 50 μg/ml). Digestion was carried out for 15 min at 37°C on a magnetic stirrer at a very low speed. After incubation, tissue was allowed to settle and the supernatant containing dissociated cells was transferred to a sterile tube. This process was repeated once and the supernatant pooled together. To inactivate the proteolytic enzymes, equal volume of 10% fetal calf serum was added to the supernatant. The cells were pelleted by centrifuging at 2000 *g* for 5 min and washed twice with culture medium. Cells isolated from one heart were plated in a flask in 6 ml of culture medium containing 5% fetal calf serum and incubated at 37°C with 5% CO_2.

2.8 *TPA treatment*

The cells were induced by the addition of TPA (in DMSO) to a final concentration of 100 ng/ml; control cultures received DMSO alone. After 24 h, RNA was isolated from the cultures following the method of Chomezynski and Sacchi (1987).

3. Results

3.1 *Expression of MLC2 and c-fos in rat heart during development and in response to pressure overload*

We first examined the expression of MLC2 and c-fos gene in the ventricles in response to pressure overload induced by aortic constriction. The coding sequence of the human cardiac MLC2 (Wadgaonkar *et al* 1993) which has 92% homology with the amino acid sequence of rat ventricular MLC2 was used as probe. The specificity of the probe was checked by Northern blot analysis. Figure 1 shows the hybridization pattern of total RNA isolated from rat heart ventricle during pressure overloaded hypertrophy. The results indicate that the expression of MLC2 is induced after 12 h of post operative period whereas that of c-fos is induced within 30 min, reaching a maximum expression of 11-fold at 6 h after aortic constriction. Thereafter the expression of c-fos begins to decline. In contrast. MLC2 expression is high only after 3 or 4 days of post operative period (28% hypertrophy) (figure 2).

Expression of c-fos gene has been shown in rat heart during early embryonic growth and normal development (Komuro *et al* 1988). To compare the expression of c-fos and MLC2 in ventricles during embryonic and normal development, the hybridization analysis using MLC2 and c-fos probes was carried out with the total RNA isolated from the hearts at different stages of embryonic and normal development. The results (figure 3) indicate a high level of expression of c-fos in both 10 and 20 days old embryonic heart that reaches a maximum of 36-fold

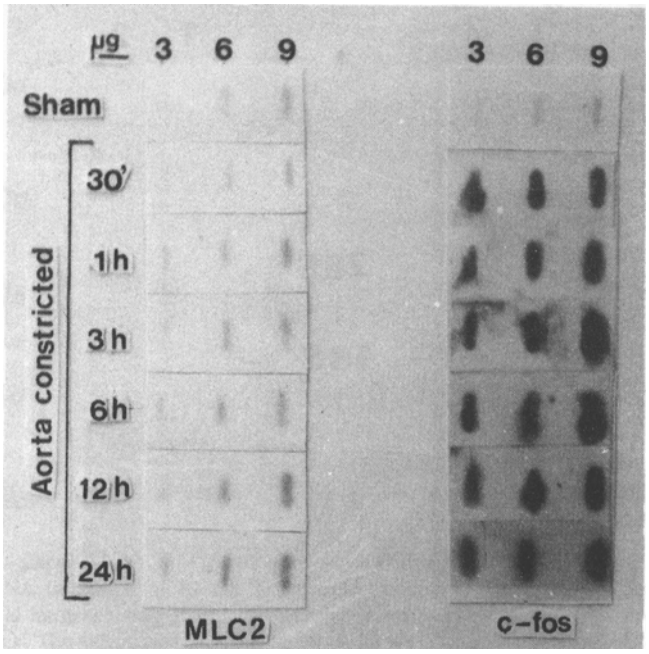

Figure 1. Expression of MLC2 and co-fos genes during development of cardiac hypertrophy in rats.

Three different concentrations (3, 6 and 9 μg) of total RNA isolated from sham-operated and aorta constricted rat hearts during early post operative period were slot-blotted and hybridized with gene specific probes. The filters were autoradiographed for 48 h at –70°C.

in 7 days old neonates when compared to the basal level expression in adult animal.

During rat heart development, the MLC2 expression is undetectable at prenatal stages. After birth, it is induced to 5-fold more in 7 days old neonates than that of basal level of expression observed at further time points. This is in contrast to c-fos expression which starts from the early stages of embryonic development. Both c-fos and MLC2 are expressed at high levels at 7 days after birth.

3.2 *Expression of MLC2 and c fos in human heart during development and cardiac abnormalities*

In order to look for the expression of MLC2 and c-fos during embryonic human heart development, we examined by slot-blot analysis, the total RNA isolated from 20, 21, 23 and 28 weeks old embryonic human heart, using the respective probes. The results indicate (figure 4) a detectable level of MLC2 expression in 20 week old embryo and a high level of expression in all the other stages analysed. Since the availability of human fetal heart samples at further stages is very limited, we analysed only these four stages. Detectable levels of expression of c-fos gene was observed in all the four different stages of embryonic human heart. While in adult

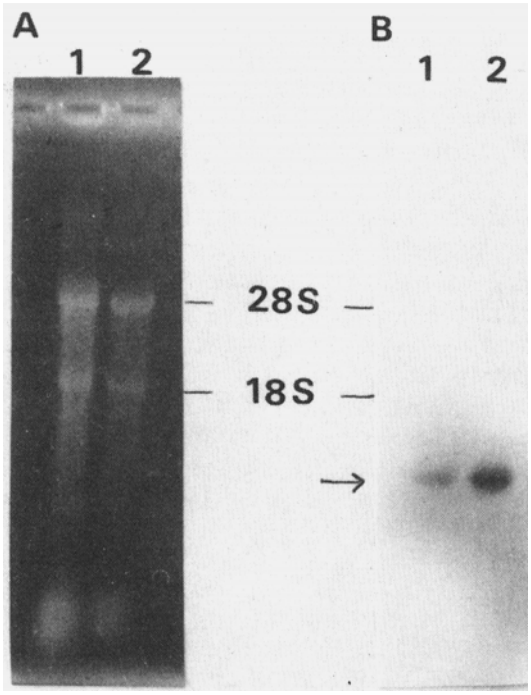

Figure 2. Northern analysis of MLC2 mRNA levels in sham-operated and 28% hypertrophied rat ventricles.

About 25 μg of total RNA isolated from sham-operated and aorta constricted (28% hypertrophy) rat ventricles were resolved on a 1·2% formaldehyde/agarose gel. It was transferred to a nylon membrane and hybridized with ^{32}P-labelled human cardiac MLC2 cDNA probe. The filter was autoradiographed for 48 h at -70°C

(**A**) Ethidium bromide stained gel. (B) Autoradiogram.

Lane I: Sham-operated; Lane 2: 28% hypertrophy; → indicates the position of MLC2 messangers.

heart, it dropped to basal level of expression. Thus, the expression of MLC2 as well as c-fos occur at considerably higher levels in the four different stages of embryonic human heart.

To check the levels of expression of MLC2 and c-fos in human hypertrophic hearts, ventricular and atrial biopsy tissues of patients with cardiac anomalies such as ventricular septal defect (VSD) with right ventricular obstruction or double chambered right ventricle [tetralogy of Fallot (TOF)] and atrial septal defect (ASD) were checked. In the ventricles and atria of these patients, accumulation of messengers for MLC2 is very high. The densitometric scanning profile of the autoradiogram revealed a maximum of 8-fold increase in MLC2 hybridization in the ASD sample while only 4-fold increase in the diseased ventricular sample. Although the level of induction vary among the atrial and ventricular tissues of the patients with ASD or TOF, it is higher than the respective normal tissue samples (figure 5). The variation of induction within the tissues may be due to variation in the degree of hypertrophy.

Similarly, in the case of c-fos also the level of expression is high in both

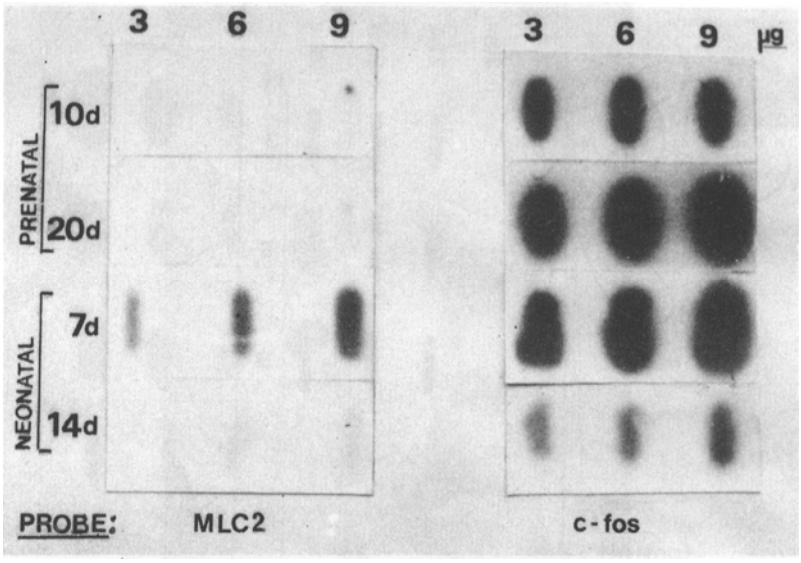

Figure 3. Expression of MLC2 and c-fos genes during rat heart development.
Total RNA isolated from 10 and 20 days old embryonic rat hearts and 7 and 14 days old neonatal rat hearts were slot-blotted and hybridized with MLC2 and c-fos RNA specific gene probes. The filters were autoradiographed for 48 h at -70°C.

ventricular and atrial tissues of cardiac patients (figure 5). The densitometry scanning of the autoradiogram revealed that the hybridization signals of ASD and VSD samples were respectively 3- to 7-fold more intense than that of control samples. Here again the intensities of signals varied between atrial and ventricular samples and the intensities of signals are high with RNA isolated from ventricular tissues of patients with TOF.

3.3 *Effect of TPA on the expression of MLC2 and c -fos in cultured hypertrophied adult cardiomyocytes*

The expression of MLC2 and c-fos in the cultured hypertrophied adult cardiomyocytes were studied in the presence and absence of a tumor promotor, 12-O-tetradecanoyl phorbol 13-acetate (TPA) (100 ng/ml). Myocytes isolated from the ventricles of normal and 28% hypertrophied rats were treated with TPA (100 ng/ml of culture medium) for 24 h. Total RNA was isolated, slot blotted and probed with c-fos and MLC2 specific gene probes. The results indicated that induced expression of MLC2 in the myocytes from 28% hypertrophied ventricle was not affected by TPA treatment in culture (figure 6). Also, it induced the expression of c-fos in both the normal and hypertrophied myocytes. These results have suggested that the induction of c-fos has no influence on the expression of MLC2 in cardiac muscle.

3.4 *Expression of c-myc and myosin heavy chain during cardiac hypertrophy*

In order to look for the expression of an another oncogene and a muscle specific gene during cardiac hypertrophy, total RNA isolated from rat hearts at different

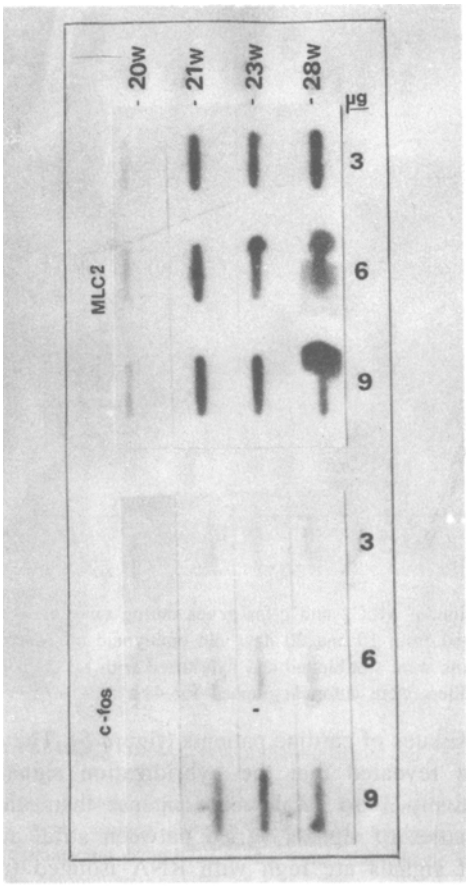

Figure 4. Expression of MLC2 and c-fos genes during human heart development.
Total RNA isolated from 20,21.23 and 28 weeks old human embryonic hearts were blotted as 3,6 and 9 µg and hybridized with ^{32}P-labelled MLC2 and c-fos RNA specific gene probes. The filters were autoradiographed for 72 h at -70°C

stages of hypertrophy were hybridized with c-myc and myosin heavy chain (MHC) specific gene probes. The results of hybridization analysis (figure 7A and 7C) showed an increased level of expression of c-myc messengers at 2 h after aortic constriction, with a drop in the expression to undetectable level at later stages of hypertrophy (40% hypertrophy). On the other hand, the accumulation of MHC messages, occurred gradually with considerably high levels at 40% of hypertrophy (figure 7B and 7C). Hybridization analysis of labelled RNA transcribed *in vitro* during transcription in isolated nuclei from hearts obtained at different stages of hypertrophy, with the above probes, showed an exactly similar pattern of increase as observed in the cytoplasmic accumulation (figure 8).

4. Discussion

Proto-oncogenes such as c-myc, c-fos and c jun have been shown to encode known or putative transcriptional factors and have been proposed to orchestrate different

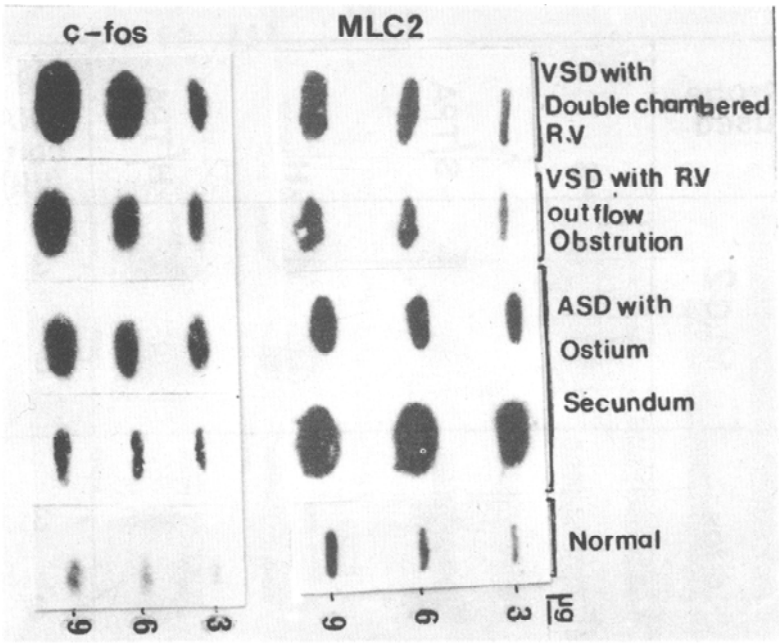

Figure 5. Expression of MLC2 and c-fos genes in atrial and ventricular tissue samples obtained from patients with cardiac disorders.

Total RNA isolated from atrial and ventricular biopsy samples obtained from patients with ASD or VSD were blotted as 3,6 and 9 µg and hybridized with MLC2 and c-fos RNA specific gene probes. The filters were autoradiographed for 48 h at -70°C.

programs of gene expression in various differentiated cell types. Expression of proto-oncogenes up or down regulates several other genes. Recent studies have implicated, the c-fos/c jun heterodimers activating phorbol inducible genes such as |human collagenase gene and human metallothionein IIA (hMTIIA) gene (Lee *et* al 1987 following binding to a consensus AP. 1 site in the promoter region of these genes. c-fos induction during seruin stimulation is mediated by serum response element (SRE) (Treisman 1986) which has homology with cArG element within the cardiac and skeletal α-actin genes that play a critical role in their muscle specific expression (Sartorelli et al 1990; Taylor *et al* 1988). In c-ras trasformed HOS cell lines the smooth muscle MLC2 expression is specifically repressed (Kumar and Chang 1992). Similarly, expression of oncogenes like v-src, v-myc, c-myc, v-erbA and .-ras can inhibit terminal differentiation of muscle cells to varying extent (Falcone et al 1985; Olson et al 1987; Payne et al 1987; Schneider *et al* 1987). In a recent report (Goswami et al 1992) the inhibition of MLC2 promoter has been attributed to the elevated levels of fos in cultured chicken cardiomyocytes. In primary skeletal muscle cells, induction of c-fos gene by phorbol ester lead to a selective disassembly of the myofibrillar apparatus and inhibition of the expression of myofibrillar genes (Cohen et al 1977). In proliferating transformed myoblast, the activated ras or fos not only inhibits muscle specific markers like MHCs, MLC but also inhibit the expression of the myoblast lineage markers such as MyoDl, MyoH and myogenin (Lassar et al 1989). In contrast, fos/jun expression in cardiac

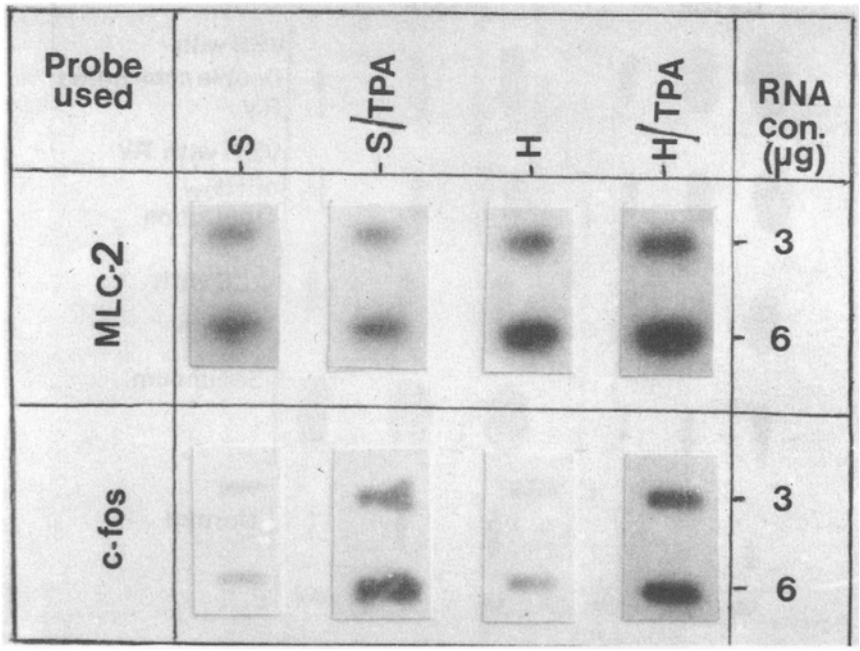

Figure 6. Slot blot hybridization analysis of MLC2 and c-fos gene expression in the cultured primary cardiomyocytes treated with TPA.

Primary cultures of cardiomyocytes isolated from the sham-operated and 28% hypertrophied rat ventricles were treated with TPA (100 ng/ml) for 24 h and the RNA was isolated. It was blotted as two different concentrations (3 and 6 µg) and hybridized with MLC2 and c-fos RNA specific gene probes. The filters were autoradiographed for 4 days at –70°C. S, Sham-operated; S/TPA, Sham-operated, TPA treated. H, 28% hypertrophy; H/TPA, 28% hypertrophy, TPA treated.

muscle cells by adrenergic agonists is associated with the assembly of MLC2 into organised contractile units (Iwake *et al* 1990). An increase in the transcription of protoncogenes like c-myc, c-fos and c-ras has been detected in the ventricle of rats during early stage of pressure overloaded hypertrophy (Izumo *et al* 1988; Komuro *et al* 1988). The upregulation of MLC2 has also been observed during hypertrophy in both human and rats (Kumar *et al* 1986; Cummins 1983). Although, the role of proto-oncogenes on the regulation of several other genes have been reported their role in the induction of contractile protein gene in cardiac muscle during normal development and hypertrophy is not known.

Our present observations indicated a low level expression of a contractile protein (MLC2 gene) gene during rat heart development. In human heart development its expression is detected as early as 20W old embryonic heart. Similarly, the c-fos expression is at detectable levels at early embryonic stages and it gradually increased during development in rat as well as in human. High level of expression of either fos or MLC2 does not exclude the expression of the other. A similar coexpression is observed in the case of rat also, as c-fos and MLC2 are expressed at maximal levels in 7 days old neonates. This may be because of cellular hypertrophy which leads to increase in the volume and myofibrillar protein content of individual

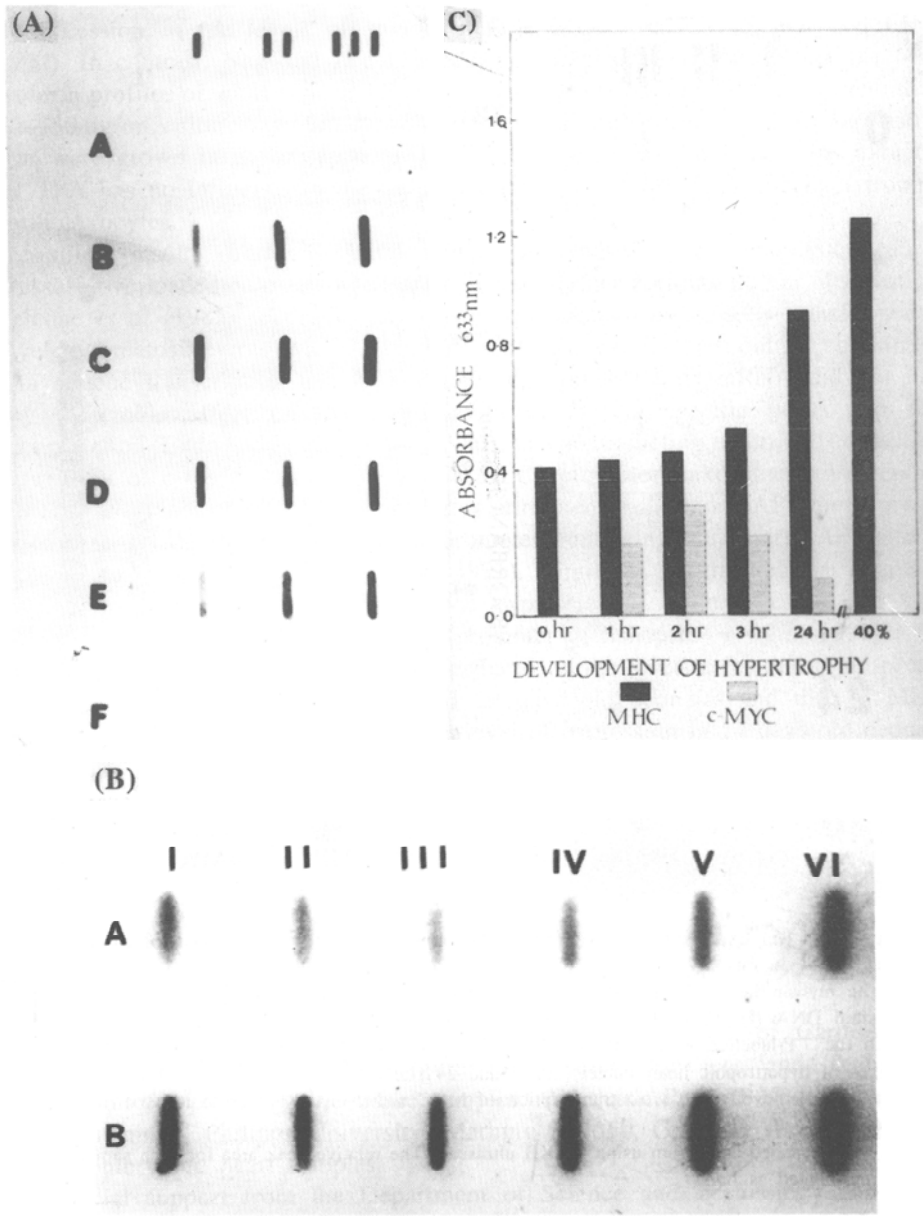

Figure 7. (A) Expression of c-myc gene during the development of cardiac hypertrophy in rats.

Total RNA isolated from sham-operated (A) and aorta constricted (B 1 h, C 2 h, D 3 h. E 24 h and F 40% hypertrophy) rat hearts were slot-blotted (I 3 μg. II 6 μg and III 9 μg) and hybridized with c-myc specific gene probe.

(**B**) Expression of MHC gene during the development of cardiac hypertrophy in rats.

Total RNA isloated from sham-operated (I) and aorta constricted (II 1 h. III 2 h, IV 3 h, V 24 h and VI 40% hypertrophy) rat hearts were slot-blotted (A 3 μg and B 6 μg) and hybridized with MHC specific gene probe.

(C) Relative accumulation of MHC and c-myc RNAs during the development of cardiac hypertrophy in rats.

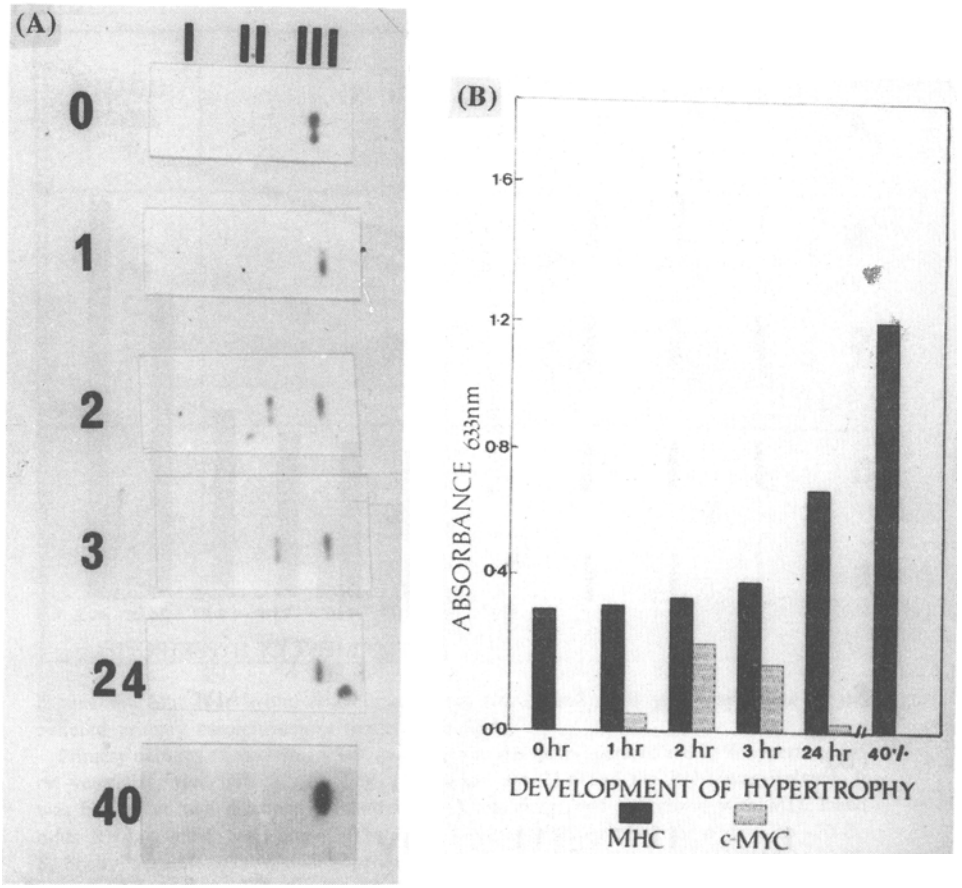

Figure 8. (A) Expression of MHC and c-myc genes during the development of cardiac hypertrophy in rats.

The myosin heavy chain (III) and c-myc cDNA (II) fragments and pBR 322 control plasmid DNA (I) (about 1 µg each) were immobilized on a nylon filter and hybridized with the 32P-labelled RNA transcribed *in vitro* from sham- operated (0h) and different stages of hypertrophic heart nuclei [1.2.3 and 24 h and 40% (12 days)].

(B) Relative level of *in vitro* transcription of MHC and c-myc RNA from isolated heart nuclei.

(A) was scanned at 633 nm using a LKB ultrasean. The relative peak area for each sample is represented as bar.

myocytes after 4th day of neonatal development (Grove *et al* 1969; Rumuyantsev 1977). Atrial and ventricular tissues obtained from human patients with cardiac disorders showed greater levels of both c-fos and MLC2 expression than their respective normal tissues.

It has been proposed that alteration of gene expression by TPA involves a cascade of events triggered by the action of protein kinase C (Nishizuka 1984) which may lead to modifications of specific transcriptional components. Expression of c-fos and activator protein 1 (API) has been reported to be modulated by TPA (Muller *et al* 1985; Lee *et al* 1987). Treatment of "HOS" cells with TPA resulted

in repression in the levels of smooth muscle MLC2 mRNA (Kumar and Chang 1992). In contrast, Nag and Lee (1992) have recently demonstrated that the MHC isoform profiles of adult ventricular cardiomyocytes exposed to TPA at concentrations 50-250 μg/mol culture medium for varying periods were similar to those of controls that were grown in the absence of TPA. We have shown here the c-fos induction by TPA has no influence in the induced expression of MLC2 in the hypertrophied cardiomyocytes.

Similar results obtained with c-myc and MHC gene expression during pressure-overloaded cardiac hypertrophy in rats, further corroborate our observations. Falcone *et al* (1985) reported that v-myc expression in avian cells transformed by myelocytomatosis virus MC29, inhibits the myogenesis. In contrast, continuous c-myc gene transcription and high cytoplasmic levels of its mRNA did not have any detectable effect on the transcription of muscle specific genes and their accumulation (Endo and Nadal-Ginard 1986). In a contradicting report, the constitutive expression of c-fos is unable to activate MLC2 promotor linked luciferase reporter genes in co-transfection studies and this is attributed to the loose API motif present in cardiac MLC2 gene. Whereas the promoter containing multimeric API sites is induced to 50-fold (Knowlton *et al* 1991). Similarly, co-transfection assays in cardiac myocytes demonstrated that over expression of c jun or c-fos plus c jun transactivated the skeletal α-actin promoter (Bishopric *et al* 1992). Our immunoprecipitation data (presented elsewhere) on the levels c-fos and c jun protein which is very high during 7 days and 20 days old neonates and that of MLC2 expression which is already at the basal level of expression in 14 days old neonates-further strengthens our view on the independent regulation of proto- oncogenes and muscle specific genes in cardiac muscles. These observations along with our present results suggest that the expression of proto-oncogenes in cardiac muscles may not necessarily be an essential requirement for muscle specific gene activation and the muscle specific genes and proto-oncogenes may be independently regulated.

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