Proto-oncogene expression in proliferating and differentiating cardiac and skeletal muscle

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We have examined the expression of 13 proto-oncogenes in proliferating and terminally differentiated cardiac and skeletal muscle. Total RNA was prepared from intact ventricular cardiac-muscle tissue and from purified ventricular cardiac-muscle cells of neonatal and adult rats and from cultured proliferating and terminally differentiated L6A1 rat skeletal-muscle cells. cDNA probes for histone H4, thymidine kinase, myosin heavy chain and M-creatine kinase were used to assess cellular proliferation and differentiation. Oncogenes *c-myc*, *c-raf*, *c-erb-A*, *c-ras-H*, *c-ski*, and *c-sis* were expressed in both proliferating and differentiated cardiac muscle tissue and cells, whereas *c-myb* expression was not observed in either. *c-src* was expressed only in neonatal cardiac muscle tissue and cells. *c-fms*, *c-abl*, and *c-ras-K* were expressed in tissue from both neonatal and adult animals but only in purified cells from neonatal animals. *c-fes/fps* was expressed only in neonatal cardiac muscles cells. *c-fos* expression was not observed in cardiac-muscle tissue from either neonatal or adult rats, but surprisingly was abundantly expressed in freshly isolated cardiac-muscle cells from animals of both ages. These results emphasize that biochemical analysis using intact cardiac-muscle tissue may not necessarily reflect muscle-specific cell processes. They also show that the expression of *c-fos* can be activated by the cell isolation procedure. *c-myc*, *c-ski*, *c-ras-H*, *c-ras-K*, *c-abl*, *c-raf* and *c-erb-A* were expressed in both proliferating and terminally differentiated skeletal-muscle cells, whereas *c-myb*, *c-fos*, *c-src* and *c-fms* transcripts were observed only in proliferating cells. *c-fes/fps* and *c-sis* were not expressed in dividing or fused skeletal-muscle cells. These results demonstrate unique tissue and cell-specific patterns of proto-oncogene expression and suggest that these genes may be involved with the regulation of cellular proliferation and terminal differentiation in striated muscle.

INTRODUCTION

The cellular homologues of acutely transforming retroviral oncogenes are termed proto-oncogenes or c-oncogenes and are thought to play an important role in the regulation of normal cellular proliferation and cellular differentiation (Bishop, 1983, 1985; Bishop *et al.*, 1985; Varmus, 1984; Weinberg, 1985). Perturbations in the expression or structure of these oncogenes are believed to be largely responsible for neoplastic transformation and growth (Bishop, 1983, 1985; Bishop *et al.*, 1985; Varmus, 1984; Weinberg, 1985). Approx. 50 different oncogenes of viral and cellular origin have been identified to date. Many of these genes can be grouped into functional classes on the basis of their effects on cellular phenotype, enzymic activity and the cellular location of their protein products. An understanding of the function of these genes in the regulation of normal cellular processes should allow us to understand better how changes in their structure and activity result in malignant transformation. In the studies reported here we have screened for the expression of 13 proto-oncogenes in two types of proliferating and differentiating striated muscle (cardiac and skeletal). Although both of these cell types are classified as striated muscle, differentiation of the cardiac-muscle cell and the skeletal-muscle cell are quite

different. The major difference is that cardiac-muscle cells are proliferating and differentiating at the same time during early development, whereas skeletal-muscle cells totally cease DNA replication and cell proliferation and then fuse into myotubes before initiating their terminal differentiation programme, i.e., in skeletal-muscle, cyto-differentiation and cellular proliferation are mutually exclusive; in cardiac muscle they are not. We initiated these studies in an attempt to correlate the expression of several proto-oncogenes with the proliferation and differentiation pattern of these two striated-muscle cell types in hopes of learning more about how these genes may be involved in the regulation of these processes.

MATERIALS AND METHODS

Preparation of cells or tissue

Adult-rat ventricular myocytes from 200–250 g-body-wt. female Sprague–Dawley rats (Holtzman) were isolated by retrograde perfusion of the hearts with collagenase (1 mg/ml) in Joklik's medium at 37 °C exactly as described (Claycomb & Palazzo, 1980; Claycomb & Lanson, 1984). The isolated cells were essentially all heart ventricular myocytes, and more than 90 % of the cells were elongated cylinders with cross-striations (Claycomb

& Palazzo, 1980). Adult-rat heart ventricular tissue was isolated from adult female Sprague-Dawley rats (about 250 g body wt.).

Neonatal-rat ventricular myocytes were isolated from 3-day-old neonatal Sprague-Dawley rats. Cells were isolated by digesting minced ventricular tissue with collagenase (0.5 mg/ml)/hyaluronidase (0.25 mg/ml) in phosphate-buffered saline exactly as described (Claycomb, 1979). Generally 100 neonatal animals were used per cell isolation. Ventricular cardiac muscle tissue was isolated from 3-day-old Sprague-Dawley neonatal rats.

L6A1 is a transformed rat skeletal-muscle clone derived from David Yaffe's original transformed L6 rat skeletal-muscle cell line (Yaffe, 1968) and was generously given by Dr. D. Ewton and Dr. J. Florini (Ewton *et al.*, 1984). These cells are able to proliferate indefinitely in proliferation media of Dulbecco's modified Eagle's medium containing 10% (v/v) horse serum, 2% (v/v) chick-embryo extract, penicillin (100 units/ml) and streptomycin (100 μ g/ml). They can be induced to differentiate terminally by placing them in fusion media of (Dulbecco medium) supplemented with 1% pre-selected horse serum, penicillin/streptomycin (as above) and 3×10^{-7} M-insulin (Eli Lilly Research Laboratories) for 8 days. On day 8, cytosine 1- β -D-arabinoside was added to a concentration of 1×10^{-5} M for 3 days.

Isolation of RNA

Total RNA was isolated by the guanidinium isothiocyanate/CsCl method essentially as described (Chirgwin *et al.*, 1979; Maniatis *et al.*, 1982). When isolating RNA from whole tissue, the freshly dissected tissue was quickly rinsed in ice-cold phosphate-buffered saline, finely minced and placed in the guanidinium solution [4 M-guanidinium isothiocyanate, 5 mM-sodium citrate (pH 7)/0.1 M- β -mercaptoethanol/0.5% sodium dodecyl sarcosinate]. It was immediately homogenized with a Brinkman Polytron instrument. Freshly isolated ventricular myocytes obtained from neonatal and adult rat hearts were pelleted in a clinical centrifuge and, after addition of the guanidinium solution, were briefly vortex-mixed. Proliferating and fused cultured L6A1 rat skeletal-muscle cells were trypsin-treated, pelleted in a table-top centrifuge and, after addition of the guanidinium solution, were briefly vortex-mixed. To every 2.5 ml of guanidinium homogenate, 1 g of CsCl was added. This was then layered over a 1.2 ml cushion of 5.7 M-CsCl/0.1 M-EDTA, pH 7.5, in Beckman SW50.1 centrifuge tubes and centrifuged at 110000 *g* at 21 °C for 16 h. The supernatant was carefully aspirated off the RNA pellet and the insides of the tube were wiped clean. The pellet was dissolved in RNA buffer [10 mM-Tris (pH 7.4)/5 mM-EDTA/1% SDS], containing 0.1 M-NaCl and 200 μ g/ml proteinase-K and incubated at 37 °C for 20 min. The solution was extracted with an equal volume of chloroform/butan-1-ol (4:1, v/v). The upper aqueous phase was saved, and the lower organic phase was extracted again with RNA buffer. These aqueous phases were pooled, and the RNA was precipitated with ethanol and pelleted by centrifuging in a Microfuge for 15 min at 4 °C. The pellet was dried briefly in a vacuum dessicator and then dissolved in RNA buffer. The quantity and purity of the RNA were determined by measuring the A_{260} and A_{280} of the solution. To this solution, 0.1 vol. of 3 M-sodium acetate,

pH 5.4, and 2.2 vol. of 95% (v/v) ethanol were added and the samples were stored at -70 °C.

RNA-denaturing gels

RNA samples were electrophoresed on a 1.2% agarose/2.2 M-formaldehyde/1 $\times$ Mops horizontal gel (10 $\times$ Mops is 200 mM-Mops/10 mM-EDTA/50 mM-sodium acetate, pH 7.0) by a modification of the procedure of Thomas (1980). Gels were run at 90 mA for approx. 6 h. During the run the gel buffer was gently recirculated with a peristaltic pump. The gel was carefully removed and placed in a tray with 1 litre of 1 $\times$ TAE [10 mM-Tris (pH 7.7)/0.5 mM-EDTA/5 mM-sodium acetate] for 15 min with gentle agitation. It was placed in 500 ml of 1 $\times$ TAE containing ethidium bromide (2 μ g/ml) and gently agitated for 15 min. The gel was destained for 30 min in 1 litre of 1 $\times$ TAE and for a further 1 h in 1 litre of fresh 1 $\times$ TAE.

Transfer to nylon membrane

Capillary transfer of the RNA from the gel to a Zeta-Probe nylon membrane (Bio-Rad) was done overnight using 1 $\times$ TAE as the transfer medium (Bio-Rad Bulletin 1110, 1985). The Zeta-Probe membrane was photographed on a u.v. transilluminator.

Hybridization and autoradiography

Conditions used for hybridization are as described by Amasino (1986), which is a modification of the method of Church & Gilbert (1984). The hybridization solution consisted of 50% (v/v) formamide, 1 mM-EDTA, 7% SDS, 0.25 M-NaHPO₄, pH 7.0, 0.25 M-NaCl and single-stranded herring testes DNA (0.1 mg/ml). All hybridizations were done at 42 °C. For non-stringent hybridizations the formamide concentration was 40%.

Probes were made by nick translation of either plasmid DNA containing the insert of interest or of gel-purified inserts cut out of the plasmid. DNA was nick-translated with [α -³²P]dATP (New England Nuclear; sp. radioactivity > 400 Ci/mmol) to a specific radioactivity of (1–2) $\times 10^8$ c.p.m./ μ g. After 1–2 days of hybridization the blots were washed three times at 62 °C in Solution I [1 mM-EDTA/40 mM NaHPO₄ (pH 7.0)/5% (w/v) SDS] and then three times at 62 °C in Solution II [1 mM-EDTA/40 mM NaHPO₄ (pH 7.0)/1% SDS] as detailed by Church & Gilbert (1984). Non-stringent washes were identical, except that they were done at 48 °C. Autoradiography was performed with Kodak X-Omat RP film and du Pont Lightning-Plus intensifying screens at -70 °C. The sizes of the transcripts were determined relative to 18 S and 28 S RNA, which were assumed to be 1.87 and 4.72 kb in length respectively.

DNA used for probes

The following plasmids or inserts cut out of plasmids were nick-translated and used as probes: thymidine kinase (1.25 kb *Bam*HI/*Sma*I human TK cDNA insert from plasmid pTK11 from Dr. Harvey Bradshaw); histone H4 (1.65 kb *Eco*RI/*Hind*III human histone H4 insert from plasmid pF0108A from Dr. Gary Stein); myosin heavy chain (1 kb *Stu*I insert for rat cardiac-muscle myosin heavy chain from plasmid pcMHC5 from Dr. Vijak Mahdavi); mouse ribosomal protein L7 (plasmid pMB9 from Dr. Robert Perry); M-creatine kinase (plasmid pCKM1 containing an insert for rat cardiac-muscle M-creatine kinase from Dr. Pamela

Benfield); *fos* (*v-fos*-specific 1 kb *Pst*I insert from plasmid pfos-1 from Dr. Inder Verma); chicken *c-myb* (1.3 kb *Hind*III/*Bam*HI insert from plasmid p-mo-myb from Dr. Diana Sheiness); chicken *v-src* (0.8 kb *Pvu*II/*Eco*RI insert from plasmid pPvuIIE from Dr. J. Michael Bishop); *sis* (1.3 kb *Pst*I insert from simian-sarcoma-virus clone C60 from Dr. Robert Gallo); *fms* (1.4 kb *Pst*I fragment of plasmid pSM3-fms from Dr. Charles Sherr); *ras* (Harvey) (0.45 kb *Eco*RI fragment of plasmid BS-9 from Dr. Ronald Ellis); *ras* (Kirsten) (1 kb *Eco*RI fragment of plasmid HiHi-3 from Dr. Ronald Ellis); *abl* (2 kb *Hind*III/*Sma*I fragment of plasmid pAB3sub3 from Dr. Stephen Goff); and *ski* (entire plasmid pSR Ski from Dr. Edward Stavnezer).

The following clones were obtained as freeze-dried bacteria carrying the plasmid of interest from the American Type Culture Collection (A.T.C.C.; Rockville, MD, U.S.A.): *erb-A* (0.45 kb *Pst*I insert from plasmid pAE Pst 0.45, A.T.C.C. no. 41018); *erb-B* (0.5 kb *Bam*HI insert from plasmid pAE Bam 0.5, A.T.C.C. no. 41021); *raf* (0.7 kb *Eco*RI/*Sal*I insert from plasmid p171, A.T.C.C. no. 41022) and chicken *fps* (1.3 kb *Eco*RI/*Hind*III insert from plasmid pfps, A.T.C.C. no. 41021).

RESULTS

Northern analysis with nylon membrane

Fig. 1 is a photograph of the nylon-membrane Zeta-Probe (Bio-Rad) after overnight capillary transfer of ethidium bromide-stained total RNA isolated from neonatal- and adult-rat cardiac-muscle cells. The u.v.-illuminated ethidium bromide-stained RNA bound to the nylon membrane is always clearer and more intense than the same ethidium bromide-stained samples in the agarose gel. Not only are the 28 S and 18 S bands clear on the membrane, but minor bands become apparent. Even after the membrane was vacuum-baked for 2 h at 80 °C, the RNA-ethidium bromide complex still fluoresced under u.v. light. We have determined that there is only a very small loss of sensitivity in the hybridization done with the RNA transferred in the presence of ethidium bromide (W. C. Claycomb & N. A. Lanson, Jr., unpublished work). In our hands, Northern analyses using nylon membranes offer significant increases in sensitivity over nitrocellulose. This procedure permits us to see the quality and the quantity of the RNA transferred and to determine if any of the RNA samples are degraded before we carry out the hybridizations. This permits a more confident interpretation of the results. As Fig. 1 shows, there is little if any degradation of the isolated RNA.

Assessment of cellular proliferation and differentiation

Fig. 2 is a composite photograph of Northern analysis of RNA of six different samples; neonatal- and adult-rat heart tissue, neonatal- and adult-rat heart cells, and cultured proliferating and fused rat L6A1 skeletal-muscle cells. To assess cellular proliferation and differentiation, cDNA probes for histone H4, thymidine kinase, myosin heavy chain and M-creatine kinase were used. Thymidine kinase and histone H4 are S-phase- and cell-cycle-expressed genes. They were used to assess the proliferative state of the tissue and cells. These two genes were expressed only in neonatal cardiac-muscle tissue

Fig. 1. Photograph of a Zeta-Probe (Bio-Rad) nylon membrane after capillary transfer of ethidium bromide-stained RNA from an agarose gel

Total RNA was extracted from 3-day-old neonatal-rat ventricular cardiac-muscle cells (lane 1) and adult rat ventricular cardiac-muscle cells (lane 2) by the guanidinium isothiocyanate/CsCl procedure and portions (15 µg/lane) were electrophoresed on a 1.2% (w/v) agarose/2.2 M-formaldehyde gel. The wet nylon membrane was photographed on a u.v. transilluminator with Polaroid type 667 film.

and cells and only in proliferating skeletal-muscle cells. It was noteworthy that, in rat RNA using a human TK probe, two transcripts of 2.6 and 1.0 kb were observed; with human RNA only one transcript of 1.5 kb is seen (Bradshaw, 1983). We have determined that, in rat, both of these RNAs are polyadenylated (W. C. Claycomb & N. A. Lanson, Jr., unpublished work); the significance of the higher- M_r transcript of thymidine kinase in rat remains to be determined. L7 is a ribosomal protein, and expression of its mRNA is not cell-cycle-dependent (Krauter *et al.*, 1979; Meyuhas & Perry, 1980; Geyer *et al.*, 1982; Jacobs *et al.*, 1985). Relatively equal levels of expression of L7 RNA were found in all samples, showing that equal amounts of RNA of each sample were loaded on to the gel (confirming the ethidium bromide staining of the RNA; Fig. 1). Probes for rat myosin heavy chain and M-creatine kinase were used to assess the expression of muscle-specific differentiation genes. These two genes are expressed in proliferating neonatal-rat heart tissue and cells and at higher levels in adult-rat heart tissue and cells. As expected, these genes were expressed only in fused, non-dividing, terminally differentiated skeletal-muscle cells.

Fig. 2. Composite of the results of Northern blot analysis of 15 µg of total RNA of six different samples [neonatal (Neo)- and adult-rat ventricular cardiac-muscle intact tissue, purified neonatal- and adult-rat ventricular cardiac-muscle cells and cultured proliferating (Prolif) and fused rat L6A1 skeletal-muscle cells]

Neonatal samples were from 3-day-old rats and adult samples were from 200–250 g female rats. The RNA was hybridized with nick-translated probes for human thymidine kinase (TK), human histone H4 (H4), mouse L7 ribosomal protein (L7), rat ventricular cardiac-muscle myosin heavy chain (MHC) and rat ventricular cardiac-muscle M-creatine kinase (M-CK). Hybridizations and washings were done under stringent conditions as detailed in the Materials and methods section. The sizes of the transcripts were determined relative to 18 S and 28 S RNA, which are assumed to be 1.87 and 4.72 kb respectively.

Screening for proto-oncogene expression

For ease of presentation, these results will be divided into three sections: neonatal- and adult-rat cardiac-muscle tissue, neonatal- and adult-rat cardiac-muscle cells, and proliferating and fused skeletal-muscle cells.

Neonatal- and adult-rat ventricular cardiac-muscle tissue. *c-myc* and *c-ski* were expressed in both neonatal and adult tissue, although at very low levels. *c-ras-H*, *c-ras-K*, *c-abl*, *c-fms*, *c-raf*, *c-erb-A* and *c-sis* were expressed to various extents in ventricular-muscle tissue from animals of both ages. *c-src* expression was observed only in neonatal-rat ventricular cardiac-muscle tissue. *c-myb*, *c-fos*, and *c-fes/fps* were not expressed in either neonatal- or adult-rat tissue.

Neonatal- and adult-rat ventricular cardiac-muscle cells. *c-myc*, *c-fos*, *c-ski*, *c-ras-H*, *c-raf*, *c-erb-A*, *c-sis* were found to be expressed in cells isolated from both neonatal- and adult-rat cardiac-muscle cells. Although *c-ski* was observed to be expressed in cells from animals of both ages, it was more abundantly expressed in the neonatal-

rat cells. It is very interesting that *c-fos* is very abundantly expressed in *isolated cells* from animals of both ages, but not at all in intact ventricular cardiac-muscle tissue. Although *c-myc* was expressed in both neonatal- and adult-rat tissue, its expression in cells isolated from animals of these ages is much stronger. *c-myb* expression is not seen in cells isolated from either neonatal or adult animals. *c-src* expression, similar to that in intact tissue, is only seen in neonatal-rat cells. *c-ras-K*, *c-abl* and *c-fms* expression, however, is observed only in neonatal-rat cardiac-muscle cells. This is in contrast with adult intact tissue, where expression of these proto-oncogenes is easily observed. Also, *c-fes/fps* expression was not detected in neonatal- or adult-rat tissue, but was seen in isolated neonatal-rat cardiac-muscle cells.

Proliferating and terminally differentiated L6A1 cultured skeletal muscle cells. *c-myc*, *c-ras-H*, *c-ras-K*, *c-raf*, and *c-erb-A* were expressed in both proliferating and fused, terminally differentiated, L6A1 cells. Expression of *c-ski*, and *c-abl* were also observed in both proliferating and fused cells; however, these two proto-oncogenes were more strongly expressed in the proliferating cells. Transcripts for *c-fes/fps*, and *c-sis* were not detected in proliferating or fused L6A1 skeletal-muscle cells. *c-myb*, *c-fos*, *c-src* and *c-fms* were observed to be expressed only in proliferating L6A1 skeletal-muscle cells.

DISCUSSION

The present study emphasizes the complexity involved in the analysis and evaluation of data concerning proto-oncogene expression and possible function in the regulation of striated-muscle cellular proliferation and differentiation. This is especially true with cardiac muscle. Intact rat ventricular cardiac-muscle tissue is heterogeneous with regard to cell type. It has been estimated that only 55% of the total cell population of the ventricular cardiac muscle of the 1-day-old rat are actually myocytes; the remaining 45% are fibroblasts, phagocytes, circulating erythrocytes and cells comprising nerve and vascular tissue (Sasaki *et al.*, 1968). Myocytes comprise only 20–25% of the total cell population of cardiac muscle of the adult rat (Morkin & Ashford, 1968; Sasaki *et al.*, 1968). Although the terminally differentiated heart myocyte in the adult animal no longer replicates DNA or divides, many of the remaining 75% of the cells that comprise the muscle tissue continue to proliferate. Therefore it is impossible, using intact heart-muscle tissue, to assign a biological parameter (such as proto-oncogene expression) to only the muscle cells. This cell-heterogeneity problem also plagues studies using neonatal-rat heart myocytes in culture. These cultures, which are usually prepared from 1–3-day-old rats, become almost completely overgrown with fibroblasts and other non-muscle cells within the first few days after plating. This occurs even when the selective-adhesion preplating technique is used (Polinger, 1970). This problem can be overcome by culturing neonatal cells in serum-free medium or culturing adult cells in the presence of cytosine 1-β-D-arabinoside (Claycomb, 1979; Claycomb & Palazzo, 1980). In the studies reported here we have attempted to circumvent the intact-tissue problem by isolating individual muscle cells, but have encountered another problem: the cell-isolation procedure apparently can activate the expression of *c-fos*

and possibly other proto-oncogenes (Fig. 3). This activation of *c-fos* may be due to the loss of cell-to-cell contact or by activation of a membrane receptor, possibly

Fig. 3. Composite of the results of Northern blot analysis of 15 µg of total RNA of the six different samples [neonatal (Neo)- and adult-rat ventricular cardiac-muscle intact tissue, purified neonatal- and adult-rat ventricular cardiac-muscle cells and proliferating (Prolif) and fused cultured rat L6A1 skeletal-muscle cells]

RNA was isolated, electrophoresed and transferred to nylon membranes as detailed in the Materials and methods section. All hybridizations and washes were done under stringent conditions, except for *fes/fps*, which was done non-stringently. The left-hand margin indicates the probe that was used and the right-hand margin shows the estimated size of the transcript(s) being expressed. The size of the transcripts were determined relative to 18 S and 28 S RNA, which are assumed to be 1.87 and 4.72 kb respectively.

by the enzymes used to isolate the cells. Another possibility is that the isolation procedure changes membrane permeability to various ions, resulting in gene activation.

The differences that we have observed in the expression of proto-oncogenes in intact cardiac tissue compared with purified cells are of interest. Several proto-oncogenes, such as *c-myc*, *c-ski* and *c-fes/fps*, appear to be expressed to greater extent in purified muscle cells than in intact tissue. Conversely, other proto-oncogenes appear to be expressed more in intact tissue than in purified cells, i.e., *c-ras-K*, *c-abl*, *c-fms*, and *c-erb-A*. These results could be due to enrichment for muscle-specific transcripts in the total RNA population extracted from purified cells. Total RNA from intact tissue would contain fewer muscle-specific transcripts on a percentage basis. Total RNA from intact tissue could contain RNA transcripts that were being highly expressed only in the non-muscle cells. Other interpretations of these data are also possible, such as the isolation procedure influencing gene expression (either activation or repression). *c-abl* expression can be used as a good example of a proto-oncogene that is expressed in adult intact muscle tissue but not in purified adult cardiac-muscle cells. This may mean that the cell-isolation procedure repressed the expression of this gene or it could mean that, in the adult, *c-abl* is expressed only in the non-muscle cells. These results re-emphasize that caution is needed when interpreting results obtained using both intact cardiac-muscle tissue and purified muscle cells. The use of cultures of neonatal- and adult-rat ventricular cardiac-muscle cells free of non-myocyte contamination as experimental systems raises the hope of lessening some of these interpretation problems. Results obtained from such culture systems could be compared with those obtained using intact tissue and purified cells in an attempt to establish if and how proto-oncogenes are involved with the regulation of cell proliferation and cell differentiation in cardiac muscle.

There have only been a few studies published concerning the expression of proto-oncogenes in differentiating skeletal muscle. *c-myc* and *c-abl* transcripts were observed to be lowered when rat L6 myoblasts reached a high cell density, stopped DNA synthesis and formed myotubes (Sejersen *et al.*, 1985). No expression of mRNA from dividing and differentiated myotube cultures was seen for *c-myc*, *c-erb-A*, *c-erb-B*, *c-mos*, *c-fes*, and *c-src*. Other investigators (Leibovitch *et al.*, 1986) looked at the expression of 15 proto-oncogenes in dividing and differentiated L6 cells. They saw detectable levels of 14 of these genes in proliferating cells. When RNA from L6 myotubes was probed, the levels of *c-abl*, *c-myc*, and *c-Ha-ras* remained unchanged, the level of *c-N-ras* was augmented, the level of *c-erb-B* was reduced, and all other c-oncogene transcripts (*c-erb-A*, *c-sis*, *c-src*, *c-fes*, *c-fms*, *c-fos*, *c-myc*, *c-Ki-ras*, and *c-fgr*) became barely, if at all, detectable. The difference among the results of each of these investigators (Sejersen *et al.*, 1985, as against Leibovitch *et al.*, 1986) and between those results and ours with skeletal-muscle cells in culture is puzzling, but may be due to differences in the cell lines used, culture conditions, serum used in the culture medium or in the sensitivity of the methods used to detect gene expression (nylon as against nitrocellulose membranes). In a detailed study (Endo & Nadal-Ginard, 1986) on *c-myc* expression in L₆E₉ skeletal-muscle cells,

it was shown that *c-myc* expression was decreased in terminally differentiated cells, but remained inducible by several growth factors, and that this regulation occurred at the transcriptional level. This means that, in skeletal-muscle cells, some genes are not irreversibly or permanently repressed during terminal differentiation. This would also appear to be true of the terminally differentiated adult cardiac-muscle cell when it is placed in culture. These adult cells were previously believed to be post-mitotic in that they had irreversibly lost the capacity to replicate DNA, undergo mitosis and divide. In the laboratory rat this loss of DNA-synthetic activity has been determined to occur by the third week of postnatal development (Rumyantsev, 1977; Claycomb, 1975, 1976, 1979, 1983). Cultured adult cells have been shown to reinitiate semi-conservative DNA replication and reacquire the activities of the enzymes needed to replicate DNA (Claycomb & Bradshaw, 1983; Claycomb & Moses, 1985). Apparently the genes coding for these enzymes are re-activated when the cells are grown in culture. Therefore, in both skeletal- and cardiac-muscle cells, some genes thought to be irreversibly repressed during terminal differentiation can be re-activated with the appropriate stimulus.

There have been two recent studies concerning oncogene expression in cardiac muscle. *c-myc* and *r-fos* expression in intact rat ventricular cardiac-muscle tissue were observed to decrease during early neonatal growth and appear to be developmentally regulated (Schneider *et al.*, 1986). Transcripts for *c-fos* were not observed by these investigators in cardiac-muscle tissue at any age. Others (Starksen *et al.*, 1986) have shown that serum, phorbol esters and noradrenaline (norepinephrine) will stimulate the expression of *c-myc* in cultured neonatal cardiac myocytes. It was suggested that this proto-oncogene may be involved with the regulation of cardiac-muscle hypertrophy (Starksen *et al.*, 1986).

The studies reported here are an initial screen for proto-oncogene expression in cardiac and skeletal muscle. Since we chose only two time periods to study (3-day-old neonatal and adult-rat cardiac-muscle and proliferating and fused skeletal-muscle cells), we may have missed important regulatory transition periods for the expression of genes involved with the control of either cell proliferation or cell differentiation. Nevertheless, we can correlate the expression of several proto-oncogenes with proliferation or differentiation events. In skeletal muscle, *c-myc*, *c-fos*, *c-src*, and *c-fms* are expressed only in dividing cells. Although *c-abl* is expressed in both dividing and fused L6 cells, its 5.9 kb transcript is much more actively expressed in the dividing cells; this is also true with *c-ski*. In cardiac muscle cells *c-ski*, *c-ras-K*, *c-src*, *c-abl*, *c-fes/fps*, and *c-fms* are expressed primarily in dividing cells. It is of interest that, as a group, proto-oncogenes which specify products with tyrosine kinase activity (*c-src*, *c-abl*, *c-fes/fps*, and *c-fms*) are expressed only in proliferating cardiac-muscle cells. Clearly, what is now needed is a systematic time study of proto-oncogene expression to establish better a possible cause-and-effect relationship for their involvement in the regulation of striated-muscle-cell proliferation and cell differentiation.

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