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Expression of c-*myc* and c-*fos* in rat skeletal muscle

Evidence for increased levels of c-*myc* mRNA during hypertrophy

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The levels of c-*myc* and c-*fos* mRNA were investigated in rat skeletal muscle by Northern hybridization. During post-natal development in the rat, c-*myc* mRNA levels were similar at birth and at 7 and 21 days of age, but then declined at 90 days and were barely detectable at 1 year. c-*fos* mRNA levels followed this pattern of expression until 90 days, but showed a large increase at 1 year. Hypertrophy of soleus and plantaris muscles was induced either by severance of the tendon to the synergistic gastrocnemius (tenotomy) or by administration of the β-adrenoceptor agonist clenbuterol. In both cases hypertrophy was associated with a rapid increase in c-*myc* mRNA levels. Following tenotomy the increase was both greater (8-fold) and more rapid (3 h) in soleus than in plantaris (2–3 fold, 12 h). Similar effects were observed during clenbuterol administration. Neither treatment caused any alteration in c-*fos* mRNA levels in the plantaris muscle. The results show that increased c-*myc* mRNA levels are an early event in the response of skeletal muscle to hypertrophic stimuli; it is argued that this occurs within the differentiated skeletal muscle fibres.

INTRODUCTION

During myogenesis, proliferating undifferentiated myoblasts fuse with one another and become terminally differentiated multinucleate myotubes, which are unable to replicate further (Nadal-Ginard, 1978). This process involves the down-regulation of genes associated with the cell cycle and the induction of muscle specific genes in a temporal and sequential pattern (Lawrence *et al.*, 1989). In the rat, myoblast proliferation (hyperplasia) happens mainly during foetal development, and the majority of muscle fibres have formed by birth (Goldspink, 1972); subsequent growth of the multinucleated differentiated myofibres occurs by increases in the cellular mass without division of the myonuclei (hypertrophy). The biochemical mechanisms involved in muscle hypertrophy are poorly understood, but in skeletal muscle it has been shown to be associated with changes in the pattern of expression of actin and myosin isoforms (Periasmy *et al.*, 1989). Similarly, in the adult rat myocardium induced to hypertrophy by pressure overload, the usually adult-muscle-specific mRNAs for α-actin and β-tropomyosin are expressed as the foetal isoforms within 2 days (Izumo *et al.*, 1988).

An earlier event in cardiac muscle hypertrophy, both in the overloaded heart and in cardiac myocytes stimulated by α-adrenergic agonists, is the expression of the proto-oncogenes c-*myc* and c-*fos* (Starksen *et al.*, 1986; Mulvagh *et al.*, 1987; Izumo *et al.*, 1988). These genes are believed to code for transcriptional regulators and have been regarded by some as exclusively involved in the regulation of events leading to mitosis and cell division (Freytag, 1988). However, particularly in the case of c-*myc*, the studies of cardiac muscle mentioned above suggest that the products of these genes may have other roles, including some functions in muscle differentiation (see Schneider & Olsen, 1988). Although c-*myc* expression is reduced during myotube formation in culture (Schneider & Olsen, 1988), this decline appears to reflect reduced cell proliferation, and it has been demonstrated that the expression of these oncogenes and muscle-specific genes is not mutually exclusive (Endo & Nadal-Ginard, 1986; Alema & Tato, 1987). Furthermore, c-*myc* expression can be stimulated in mature myotubes (Endo & Nadal-Ginard, 1986). In particular, the results from the study of cardiac hypertrophy suggest that changes in expression of c-*myc* and c-*fos* are part of a sequence of events which lead to cardiac cell hypertrophy (Izumo *et al.*, 1988).

The aims of the present work were to investigate the expression of c-*myc* and c-*fos* in skeletal muscle by measuring the levels of c-*myc* and c-*fos* mRNA during post-natal development and hypertrophy. The study employed Northern hybridization and two models of skeletal muscle hypertrophy, one induced by compensatory workload after severance of the tendon to a synergistic muscle, and the other induced pharmacologically using the β-adrenoceptor agonist clenbuterol.

MATERIALS AND METHODS

Chemicals and DNA probes

The c-*myc* probe, a gift from Dr. M. Cole, Princeton University, NJ, U.S.A., was the cDNA of the 3 exons of the mouse c-*myc* gene. The 2.3 kbp cDNA was cloned into pT7 and a 1.8 kbp *Hin*dIII fragment was used in hybridization studies. The v-*fos* probe, a 1.0 kbp *Pst*1 fragment cloned into pBR322 (Curran *et al.*, 1982), was a gift from Dr. G. Birnie, Beatson Institute, Glasgow, U.K. A probe to 18 S rRNA (Erikson *et al.*, 1981) was kindly donated by Dr. R. Fulton (also of the Beatson Institute). Multiprime labelling kits, Hyperfilm-MP and [^{32}P]dCTP were purchased from Amersham International, Amersham, Bucks., UK. Genescreen nylon membrane was purchased from NEN Dupont, and chemicals were either AnalaR or molecular biology grade.

Animals and experimental procedures

Hooded Lister rats of the Rowett strain were used throughout. In studies of muscle development, animals were weaned at 19 days old and fed on stock diet (CRM nuts, Labsure; K+K Greff, Croydon, U.K.) *ad libitum*.

Compensatory hypertrophy was induced in young male rats (30 days old) by tenotomy under ether anaesthesia. A small incision was made in the skin of the right leg, and the tendon to the gastrocnemius muscle was separated carefully from those to the soleus and plantaris, using blunt forceps. The distal tendon of the right gastrocnemius was then sectioned and the skin

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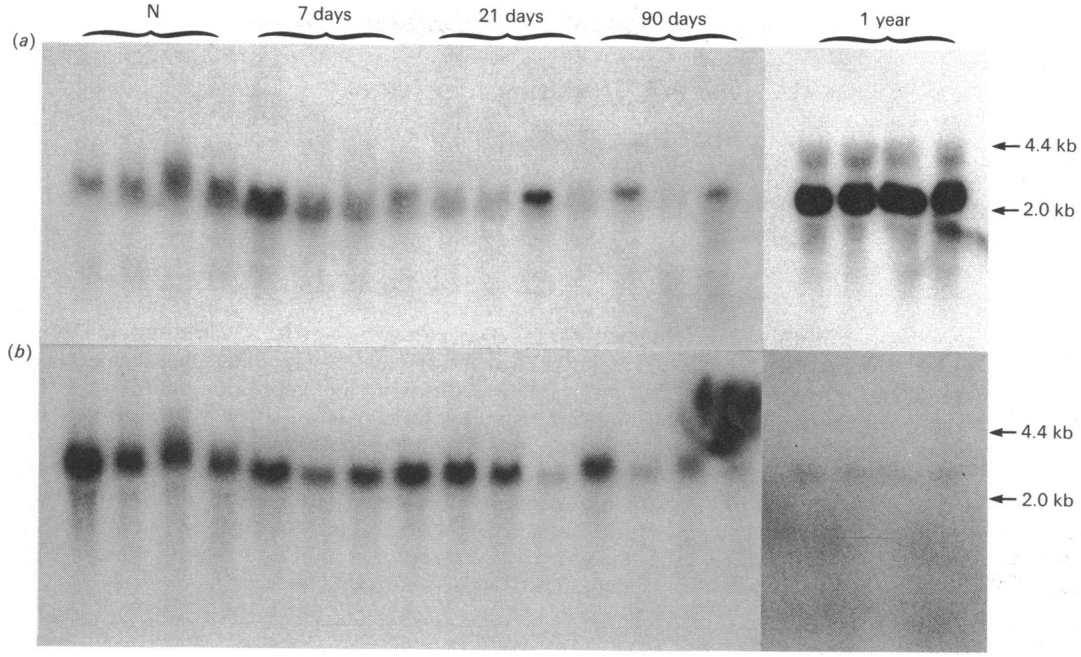

Fig. 1. Northern hybridization showing the levels of c-*fos* (*a*) and c-*myc* (*b*) mRNAs in skeletal muscle from female rats of different ages

All lanes were loaded with 25 μg of total RNA and filters were hybridized successively with c-*myc*, v-*fos* and 18 S rRNA probes. Results show levels of c-*fos* (*a*) and c-*myc* (*b*) mRNAs detected by autoradiography. For a given probe all samples were hybridized under identical conditions. Samples were taken from whole leg muscle of neonatal (N) and 7-day-old rats, and from the gastrocnemius muscle for 21-day-, 90-day- and 1-year-old animals (four lanes for each time point; each lane represents a different RNA preparation from a different animal).

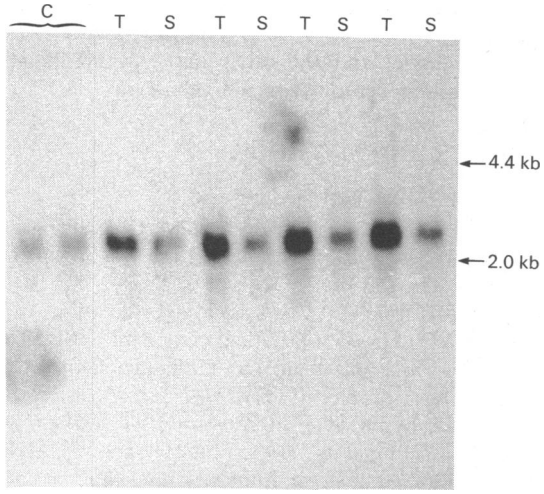

Fig. 2. Northern hybridization showing levels of c-*myc* mRNA in the hypertrophying soleus after severance of the tendon to the gastrocnemius (tenotomy)

Results show detection of c-*myc* mRNA by autoradiography following hybridization with a probe to the three exons of the mouse c-*myc* gene. Hybridization was to total RNA from unoperated controls (C, $n = 2$), sham-operated controls (S, $n = 4$) and tenotomized animals (T, $n = 4$) 12 h after operation. All lanes were loaded with 25 μg of total RNA.

resealed with two stitches. Sham operations were performed on the left legs, which were subjected to the same surgical procedure but without cutting the tendon. The animals regained normal gait within minutes and use of the right limb was not apparently impaired by the surgery. A further six animals were designated as unoperated controls. At various times after the operation the animals were killed, and the soleus and plantaris muscles were removed and rapidly frozen in liquid N_2 and stored at -70 °C until analysed. Samples from other experiments were treated similarly.

In further experiments, hypertrophy was induced by the β-adrenoceptor agonist clenbuterol. At 27 days old, male rats were fed a semi-synthetic diet (PW3; Pullar & Webster, 1977) *ad libitum* for 3 days. The animals were then split into two groups, one fed on PW3 diet only (controls) and the other fed on PW3 containing clenbuterol (2 mg/kg) (Maltin *et al.*, 1986). In experiments when very early effects (3–6 h) were investigated, clenbuterol was administered orally in a dose equivalent to that which would have been ingested in 3–6 h assuming a daily intake of 10 g of food.

RNA extraction and hybridization

The soleus and plantaris muscles were broken up while frozen and then homogenized in 4 M-guanidinium thiocyanate/25 mM-sodium citrate (pH 7.0)/0.5 % sarkosyl/0.1 M-2-mercaptoethanol (1 ml/100 mg of muscle) using an Ultra-Turrax. Subsequent extraction of total RNA followed the acid/guanidinium/chloroform procedure of Chomczynski & Sacchi (1987), proportionally scaled down into microcentrifuge tubes for samples weighing less than 100 mg. RNA species were then separated by electrophoresis through a denaturing 2.2 M-formaldehyde/1.2 % agarose gel (Maniatis *et al.*, 1982) and transferred to a nylon membrane (Genescreen) by capillary blotting. RNA was fixed to the membrane by exposure to u.v. light and the membranes were stored dry until required. Membranes were pre-hybridized overnight at 42 °C with denatured salmon sperm DNA (0.1 mg/ml) in 50 % formamide, 10 % dextran sulphate, 0.2 % BSA, 0.2 % polyvinylpyrrolidone, 0.2 % Ficoll, 0.1 % sodium pyrophosphate, 1 % SDS and 50 mM-Tris/HCl, pH 7.5.

DNA probes (50–100 ng), which had previously been re-

stricted and separated by electrophoresis in a 0.6% low-melting-point agarose gel (Gibco-BRL), were labelled with [^{32}P]dCTP by random priming, and the labelled DNA was then separated from free nucleotides by gel filtration on Sephadex G-50; probe specific radioactivities were approx. 10^9 c.p.m./μg of DNA. The labelled probes were added to the prehybridization mixture and hybridized at 42 °C for 24 h. The membranes were washed to remove non-specific hybridizations, twice in 2 × SSC (1 × SSC = 0.15 M-NaCl/0.015 M-sodium citrate) at room temperature for 5 min, followed by 0.5 × SSC/1% SDS at 65 °C for 1 h (twice). Specific hybridization was then detected by autoradiography using Hyperfilm-MP at −70 °C (up to 1 week for c-*myc*). After autoradiography, membranes were washed in 0.1% SDS for 5–7 min at 95 °C before rehybridization to other probes.

The absorbance of the bands on the film was quantified using a QUIPS image processing work station (Torch Computers, Cambridge, U.K.) operating with VCS image processing software (Vision Dynamics, Hemel Hempstead, Herts., U.K.). Autoradiographs were placed on a photographic light box and images were captured with a Hitachi KP 140 CCD camera (resolution 768 × 256 pixels, at 256 grey levels). The level of expression of the c-*myc* or c-*fos* mRNA was calculated as the ratio of the intensity of the band after hybridization to the intensity of the band after subsequent hybridization with the probe to the 18 S rRNA; this presents the level of specific mRNA per unit rRNA, and minimizes any variation in loading of the gel. The ratios were then expressed as percentages of the mean value for control tissues on each filter; this enabled direct comparison between filters. Most data were analysed using a two-tailed Student's *t* test. However, the within-filter variation found in the clenbuterol experiment required a more sophisticated treatment in which data from both groups of animals were subjected to an analysis of variance such that bias, in the form of quadratic trends arising from the position of the signal on the filter, was eliminated from the analysis.

RESULTS

Hybridization of Northern blots showed that both the c-*myc* and c-*fos* probes reacted with single RNA species in preparations of total RNA from skeletal muscle (Fig. 1); the sizes of the two RNAs detected (2.4 and 2.2 kb) corresponded to the known sizes of the c-*myc* and c-*fos* mRNAs.

The levels of proto-oncogene mRNA were investigated during the post-natal development of the rat by Northern blotting and hybridizations on total RNA from the muscle of neonatal rats and those aged 7 days, 21 days, 90 days and 1 year. In the neonatal and 7-day-old animals, muscle was taken from the whole leg for analysis, while the gastrocnemius was used from the older animals (females). c-*myc* mRNA levels were initially relatively high in the neonate and remained so until 21 days of age (Fig. 1). Thereafter the mRNA declined with age until barely detectable in the 1-year-old female rat. The c-*fos* mRNA levels followed this pattern of expression from the neonate until 90 days old, but the 1-year-old female rats showed a very large increase in the amount of c-*fos* mRNA (Fig. 1).

The early responses to the hypertrophic stimulus of compensatory work overload were investigated 3, 6, 12, 24 and 48 h after tenotomy. Visual inspection of the autoradiographs suggested that the induced workload produced an increase in the amount of c-*myc* mRNA in both the soleus and plantaris muscles (for example, results for soleus muscle 12 h after tenotomy are shown in Fig. 2). Quantification of the extent of hybridization (Fig. 3) showed that both the time course and magnitude of the changes in c-*myc* expression were different in the two muscles. Thus the soleus muscle responded more rapidly; the c-*myc* mRNA levels were significantly increased 3 h after tenotomy in the soleus, but not until 12 h in the plantaris. Similarly, the soleus was also more sensitive, as shown by the 7–8-fold increase

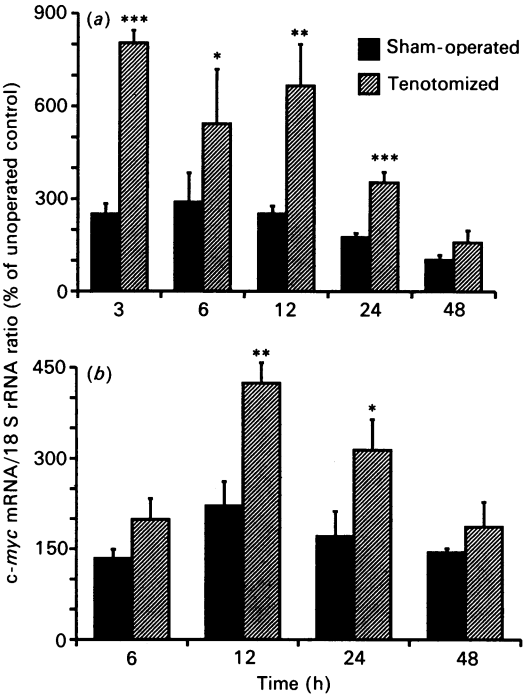

Fig. 3. Quantification of changes in the c-*myc* mRNA/18 S rRNA ratio during hypertrophy of soleus (*a*) and plantaris (*b*) muscles following tenotomy

Samples of total RNA were loaded on to duplicate filters and all were hybridized under identical conditions. Filters were hybridized successively with c-*myc* and 18 S probes and the degree of hybridization was detected by autoradiography. After quantification of the autoradiograms by image analysis, the ratios of hybridization with the c-*myc* probe to that with the 18 S probe were calculated, and average values from the duplicate filters were expressed as percentages of values obtained for unoperated controls. Means ± S.E.M. were calculated from groups of five animals and compared using Student's *t* test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicate significant differences from sham-operated groups.

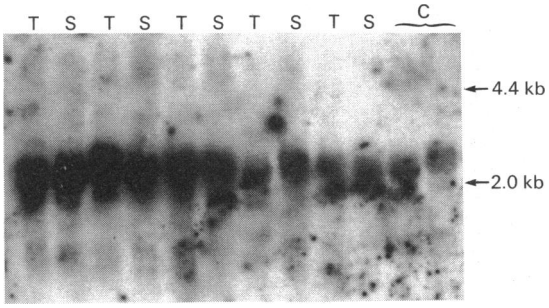

Fig. 4. Northern hybridization showing levels of c-*fos* mRNA in the hypertrophying plantaris after severance of the tendon to the gastrocnemius (tenotomy)

Results show detection of c-*fos* mRNA by autoradiography following hybridization with a probe to the three exons of the mouse v-*fos* gene. Hybridization was to total RNA from controls (C, $n = 2$), sham-operated (S, $n = 5$) and tenotomized (T, $n = 5$) animals 12 h after operation. All lanes were loaded with 25 μg of total RNA.

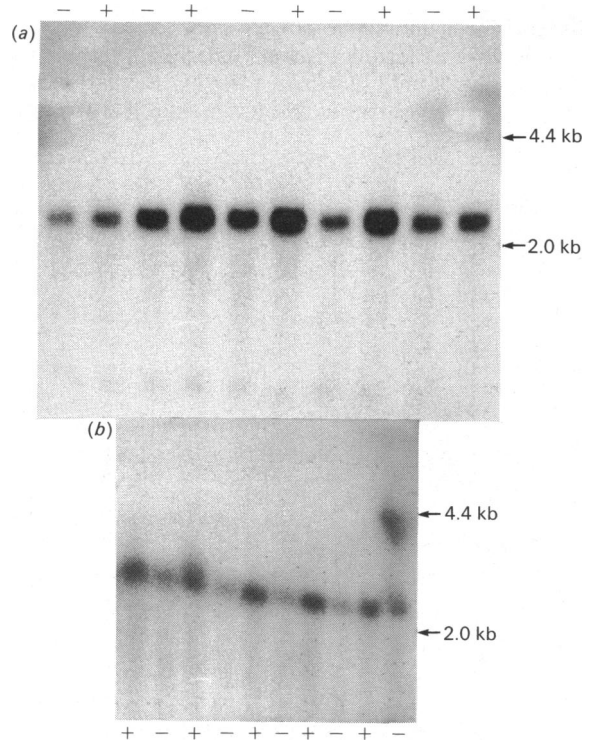

Fig. 5. Northern hybridizations showing the effect of clenbuterol on the level of c-*myc* mRNA in soleus (*a*) and plantaris (*b*) muscles

Results show detection of c-*myc* mRNA by autoradiography following hybridization with a probe to the three exons of the mouse c-*myc* gene. Lanes were loaded with 25 μg of total RNA from control (−) or clenbuterol-treated (+) animals. Results are shown for muscles taken after 24 h for soleus and 6 h for plantaris.

detected in the soleus (compared with unoperated controls) and the 2–3-fold increase in plantaris. The maximum effect was observed at 3 h in soleus and 24 h in plantaris, after which times the c-*myc* mRNA levels returned towards control levels. In sham-operated controls both muscles showed very much smaller increases in c-*myc* mRNA (20–150 % in soleus and 20–30 % in plantaris). Rehybridization of filters with the v-*fos* probe showed no difference the c-*fos* mRNA levels between the plantaris muscles from the control, sham-operated and tenotomized animals (Fig. 4).

The expression of c-*myc* and c-*fos* during early stages of hypertrophy were also investigated in the plantaris and soleus muscles of rats given clenbuterol. As after tenotomy, the hypertrophy of both soleus and plantaris muscles induced by clenbuterol was associated with a significant and rapid increase in the level of c-*myc* mRNA (Fig. 5). The variation in hybridization achieved with these filters made it impossible to study the time-related changes in c-*myc* mRNA after clenbuterol administration. However, formal statistical treatment of the data showed that at certain time points (3–24 h in soleus; 6 h in plantaris) clenbuterol did increase c-*myc* mRNA levels ($P < 0.01$). There was no evidence of a change in the c-*fos* levels in the plantaris muscle following the administration of clenbuterol (results not shown).

DISCUSSION

Hybridization of Northern blots of muscle RNA showed that both c-*myc* and c-*fos* mRNAs are present in skeletal muscle. Both mRNAs were present in greatest amounts during neonatal and early life, decreased after weaning at 21 days and remained at these lower levels until the animal was fully grown and sexually mature (90 days). The decline in c-*myc* expression continued into old age and was barely detectable in 1-year-old female rats which had stopped growing. The higher expression in muscle from neonatal and pre-weaning rats compared with 90-day-old rats suggests that the higher c-*myc* levels occur during the period when there is rapid muscle growth by enlargement of existing fibres. The subsequent decline may reflect either falling expression within myofibres or a gradual decrease in the number of satellite cells in the muscle. c-*fos* mRNA levels were also low in the 90-day-old rat, but in contrast increased markedly in 1-year-old female animals. A similar increase in c-*fos* expression in cardiac muscle from rats of 200 days of age has been reported (Komuro *et al.*, 1988); however, at present there is no explanation for these findings. Both c-*myc* and c-*fos* have been observed previously to be expressed in muscle cell cultures (Claycomb & Lanson, 1987; Schneider & Olsen, 1988) and heart tissue, but in the case of c-*fos* the culture process was observed to induce expression (Claycomb & Lanson, 1987). The present results show that these two proto-oncogenes are also expressed in post-natal skeletal muscle tissue.

Furthermore, the level of c-*myc* mRNA was stimulated 2–8-fold during muscle hypertrophy induced either by severance of the tendon to a synergistic muscle or by clenbuterol. The effects appeared to be independent of muscle type, since they occurred in both the soleus (tonic, largely slow fibres) and plantaris (phasic, largely fast fibres) muscles. The stimulation seen in the tenotomy experiments could theoretically be due to an increase in c-*myc* mRNA levels in the myofibres, in satellite cells, or in lymphocytes and macrophages induced to proliferate by post-operative inflammation. However, there are several arguments which suggest that the observed increase in c-*myc* mRNA levels reflects increases within the muscle fibres themselves rather than in satellite cells or proliferating cells of non-myoblast origin. Firstly, the increase seen following tenotomy was much larger than that seen in sham-operated animals compared with unoperated controls. It would thus appear that although lymphatic cell infiltration may account for the increase observed in the sham-operated animals, it cannot explain the large increase in tenotomy.

This view is supported by the observation that in four animals in which complete tenotomy (i.e. severance of tendons to soleus, plantaris and gastrocnemius muscles) was carried out, and in which therefore the soleus and plantaris were not stimulated to hypertrophy, the increases in c-*myc* mRNA were within the sham levels. For example, in the soleus at 3–12 h after operation the c-*myc* mRNA/18S rRNA ratio was 171 ± 46 ($n = 4$) compared with a value of 100 for the unoperated controls. In addition, it is unlikely that the differential expression of c-*myc* mRNA in soleus muscle compared with plantaris muscle could be explained by inflammatory responses. Lastly, c-*myc* mRNA levels were also increased in hypertrophy induced by the non-surgical method of clenbuterol administration. Since satellite cells account for such a small proportion (2–10 %) of myonuclei, it is highly improbable that the 2–8-fold increase in c-*myc* induced within 3–12 h was due to expression in activated satellite cells. Satellite cell activation would be expected to be associated with increased c-*fos* mRNA levels, so the observed lack of change in c-*fos* in these experiments also suggests that there was not sufficient activation of satellite cells to account for the increases in c-*myc* mRNA. We conclude, therefore, that the increased level of c-*myc* mRNA observed in hypertrophying muscle reflects increased levels within the skeletal muscle fibres. This is consistent with the facts that (1) c-*myc* mRNA levels change in cardiac hypertrophy although there are reportedly no satellite cells in the heart (Zak,

1973), and (2) c-*myc* expression can be induced in myotubes in culture (Endo & Nadal-Ginard, 1986).

The increase in c-*myc* mRNA observed in hypertrophying skeletal muscle is comparable with the increases found in the heart under pressure overload (Izumo *et al.*, 1988; Komuro *et al.*, 1988) and in stimulated cardiomyocytes (Starksen *et al.*, 1986); however, in contrast to these earlier studies, the present studies showed no change in c-*fos* mRNA levels in the plantaris muscle. In the present work, the lack of change of c-*fos* shows that, at least in the case of the plantaris muscle, the increase in c-*myc* does not simply reflect a general increase in mRNA synthesis but is a specific response. In quiescent undifferentiated cells c-*myc* mRNA is one of the early genes to be expressed in response to a mitotic stimulus (Lau & Nathans, 1985), and in cardiac muscle the induction of a pressure overload results in elevated expression within 3 h (Izumo *et al.*, 1988). The time course of the changes induced in both soleus and plantaris muscles suggest that an increase in c-*myc* mRNA is an early occurrence in a sequence of events that is elicited by stimulation of skeletal muscles to hypertrophy; the increase at 3–6 h in soleus precedes increases in muscle protein synthesis and total RNA (Goldberg *et al.*, 1975) and elevation of insulin-like growth factor mRNA levels (De Vol *et al.*, 1990). At present it is unclear whether the increase in c-*myc* mRNA levels is due to increased gene transcription, increased stability of the mRNA, or a combination of both.

It has been suggested (Goldberg *et al.*, 1975) that in hypertrophy induced by tenotomy the soleus is subjected to a greater workload than the plantaris, and this is reflected in an initially increased rate of muscle growth for the soleus (Morgan & Loughna, 1989). This may account for the increased sensitivity of the soleus muscle in both the magnitude and speed at which c-*myc* mRNA levels are elevated when compared with the plantaris. Alternatively, the constant innervation of the tonic soleus muscle (similar to the heart in this respect) may accentuate and accelerate the response induced by tenotomy.

The importance of c-*myc* in the control of muscle gene expression is illustrated by three observations: (1) the *myc* protein has regions of sequence similarity with skeletal myogenic factors such as *myoD1* (Tapscott *et al.*, 1988; Murre *et al.*, 1989), (2) transgenic animals expressing excess c-*myc* show increases in heart size (Jackson *et al.*, 1990), and (3) cardiac hypertrophy is associated with transient increases in c-*myc* mRNA levels (Mulvagh *et al.*, 1987; Izumo *et al.*, 1988; Komuro *et al.*, 1988). The observed increases in c-*myc* mRNA levels during skeletal muscle hypertrophy support the view that c-*myc* has an important role in the control of muscle cell growth. They also support the concept that c-*myc* expression can be induced in terminally differentiated myocytes (Endo & Nadal-Ginard, 1986) and is not exclusively associated with cell proliferation. In conclusion, the increase in c-*myc* mRNA appears to be an early event in the response of both skeletal and cardiac muscle to hypertrophic stimuli. This suggests that it may trigger a cascade of events, possibly including activation of insulin-like growth factor genes, which can lead to cell growth and hypertrophy in terminally differentiated cells such as those of skeletal and cardiac muscle.

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