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Differential regulation of actin and myosin isoenzyme synthesis in functionally overloaded skeletal muscle

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Overload hypertrophy of the chicken anterior latissimus dorsi muscle is accompanied by a replacement of one myosin isoenzyme (slow myosin-1, SM1) by another (slow myosin-2, SM2). To investigate the molecular mechanisms by which these changes occur, we measured the fractional synthesis rates (k_s) *in vivo* of individual myosin-heavy-chain isoenzymes, total actin and total protein during the first 72 h of muscle growth. Although the k_s of total protein and actin were doubled at 24 h, the k_s for SM1 and SM2 were depressed. However, the k_s of both isomyosins were nearly tripled by 72 h. Despite the increase in muscle size observed at 72 h, the amount of SM1 was reduced by half, indicating increased degradation of SM1. Results of translation of polyribosomes *in vitro* paralleled the results obtained *in vivo*. The proportion of total polyadenylylated mRNA in total RNA was increased at 48 and 72 h, but unchanged at 24 h despite the increase in protein synthesis at 24 h. Nuclease-protection analyses indicate that the level of specific SM1 and SM2 mRNAs change in a reciprocal fashion during overload. We conclude that gene-specific and temporal differences exist in the regulatory mechanisms that control overload-induced muscle growth.

INTRODUCTION

The dramatic increase in size that occurs in overload hypertrophy of skeletal muscle is accompanied by a change in the repertoire of genes expressed (Gutmann *et al.*, 1970; Holly *et al.*, 1980; Gregory *et al.*, 1986, 1987*a*; Tsika *et al.*, 1987; Periasamy *et al.*, 1989). In the chicken ALD muscle the normal developmental replacement of one myosin isoenzyme (slow myosin-1, SM1) by another (slow myosin-2, SM2) is greatly accelerated by the attachment of a weight to the wing (Kennedy *et al.*, 1986).

An increase in the rate of total protein synthesis and degradation, as well as an increase in total RNA concentration, appear to be involved in muscle hypertrophy (Laurent *et al.*, 1978; Goldspink *et al.*, 1983). However, the mechanisms of protein isoform transitions are unknown. In the mammalian heart, the α-to-β-MHC transition that accompanies ventricular hypertrophy induced by pressure overload appears to occur by a pre-translationally controlled increase in the synthesis rate of the β-MHC (Lompre *et al.*, 1984; Nagai *et al.*, 1987). On the other hand, a pre-translationally controlled increase in the synthesis rate of the α-MHC, along with a concurrent decrease in the β-MHC synthesis rate, has been reported to occur in response to thyroid hormone (Everett *et al.*, 1984). As far as other proteins are concerned, during recovery of atrophied skeletal muscle the synthesis of actin appears to be controlled initially at pre-translational levels, and later by translational mechanisms (Morrison *et al.*, 1986). However, the regulatory mechanisms controlling the synthesis of cytochrome *c* may be different (Morrison *et al.*, 1987).

In the present study we have investigated the molecular mechanisms responsible for the myosin isoenzyme transition in the overloaded chicken ALD muscle. Synthesis rates of individual myosin isoenzymes, total actin and total protein were determined *in vivo* during the first 72 h of muscle growth, and the relative abundance of total and isoform-specific messenger RNAs was measured. The results suggest a complex scheme of regulation which includes independent regulation of the synthesis of individual myosin isoenzymes, non-co-ordinate regulation of contractile-protein synthesis, and different regulatory mechanisms in the initial and later phases of overload.

Some of the results of this investigation have already been presented in a preliminary form (Gregory *et al.*, 1987*b*).

METHODS

Animal model and infusion of isotope

A total of 19 5-week-old male White Leghorn chickens (Spafas, Norwich, CT, U.S.A.) were allowed access to

* Abbreviations used: ALD, anterior latissimus dorsi; PLD, posterior latissimus dorsi; SM1, slow myosin-1; SM2, slow myosin-2; PAGE, polyacrylamide-gel electrophoresis; k_s, fractional synthesis rate; k_d, fractional degradation rate; k_g, fractional growth rate; S.A., specific radioactivity; MHC, myosin heavy chain; nt, nucleotide.

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food and water *ad libitum*. A lead weight, wrapped in gauze and equivalent to 10% of the body weight, was attached to the humerus of one wing as described previously (Sola *et al.*, 1973; Kennedy *et al.*, 1986). The contralateral wing served as a control for all measurements. After 24, 48 or 72 h the animals were injected intravenously with a flooding dose (150 μmol/100 g body wt.; Garlick *et al.*, 1980) of L-[4-^{3}H]phenylalanine (Amersham Corp.) containing either 5 or 10 mCi. The injection was completed within 40 s. Blood samples were withdrawn at several time points during the experiment for the determination of plasma specific radioactivity (S.A.) of [^{3}H]phenylalanine. After 20 or 40 min the animals were killed by Nembutal overdose, the muscles quickly removed, frozen on solid-CO_2 blocks, and stored at −80 °C for later analyses. The water content of control and overloaded ALD muscles was determined on a separate group of 15 animals by drying the muscle to constant weight in a 110 °C oven.

Analysis of myosin isoenzyme content

The proportions of myosin isoenzymes were analysed by pyrophosphate PAGE as described previously (Hoh *et al.*, 1976; Everett *et al.*, 1983). Gels were run at 160 mA constant current for 19–24 h. The gels were stained with 0.04% Coomassie Brilliant Blue G-250 in 3.5% (w/v) $HClO_4$ (Reisner *et al.*, 1976). Under these conditions the gels did not require destaining. The relative amounts of SM1 and SM2 were determined by scanning densitometry, and the areas under the peaks were integrated (Quick-Scan, Jr. equipped with a Quick Quant III integrator; Helena Laboratories, Beaumont, TX, U.S.A.).

Separation of myosin isoenzymes and synthesis-rate measurements

The two myosin isoenzymes of the ALD muscle, SM1 and SM2, were separated by immunoadsorbent chromatography from an extract of total myosin. Muscles were homogenized in a low-salt buffer (40 mM-NaCl/5 mM-Na_2HPO_4/5mM-NaH_2PO_4/1mM-$MgCl_2$/0.1mM-dithiothreitol/0.1 mM-EDTA, pH 7), centrifuged, and the supernatant saved for determination of the S.A. of the intracellular free pool of [^{3}H]phenylalanine. The pellet was washed twice with the low-salt buffer to remove the bulk of sarcoplasmic proteins. The pellet was then dissolved in the myosin extraction buffer (0.3 M-NaCl/0.1 M-NaH_2PO_4/0.05 M-Na_2HPO_4/1 mM-EDTA/1 mM-ATP, pH 6.5), left on ice for 30 min, centrifuged, and 10 vol. of cold distilled water added to the supernatant to precipitate the myosin overnight. After centrifugation at 10000 ***g*** the pellet was dissolved in 1 ml of column buffer (0.5 M-NaCl/20 mM-NaH_2PO_4/20 mM-Na_2HPO_4, pH 7.4) and added to a tube containing 1 ml (packed volume) of CNBr-activated Sepharose 4B beads (Pharmacia) to which monoclonal antibody CCM-52 (Clark *et al.*, 1982) had been coupled in accordance with the manufacturer's instructions. This antibody binds to the SM1 isoform, but not to SM2 (Kennedy *et al.*, 1986). The tube was rotated gently overnight at 4 °C, centrifuged, and the supernatant containing SM2 was removed. The beads were washed twice with column buffer and the washes added to the supernatant, which was then filtered through glass wool to remove fines, dialysed against 0.3 M-NaCl/0.125 M-Tris buffer, pH 6.8, and concentrated to a volume of less than 1 ml with Ficoll 400 (Pharmacia). After washing the beads three times more with column buffer to remove (and discard) any remaining SM2, and three times with 0.4 M-$MgCl_2$/20 mM-Tris, pH 7.5, the bound SM1 was released from the beads by the addition of 4 M-$MgCl_2$/20 mM-Tris, pH 7.5, dialysed against 0.15 M-NaCl/0.125 M-Tris, pH 6.8, and concentrated as described for SM2. A small amount of each purified isoenzyme was reserved for SDS/-polyacrylamide slab-gel electrophoresis (Carraro & Catani, 1983) to assess the purity of the isoenzyme preparation (Fig. 2 below). MHCs were then separated from light chains by subjecting the remainder of the isoenzyme preparation to tube SDS/PAGE (Weber & Osborn, 1969), and slicing the MHC band from the gel with a razor blade. Separate SDS/PAGE gels of total protein extracts were run for measurement of total MHC and actin-synthesis rates. Isolated gel bands containing total MHC, individual isoenzymes or actin were hydrolysed in 6 M-HCl at 110 °C for 24 h. The hydrolysate was evaporated to dryness (Evapomix; Buchler Instruments, Fairfield, NJ, U.S.A.), redissolved in distilled water, and passed over an ion-exchange column (Dowex AG 50W-X4) to remove acrylamide contaminants. After elution with aq. 25% (v/v) NH_3, the eluate was dried as described above and redissolved in distilled water. The [^{3}H]phenylalanine S.A. (d.p.m./pmol) in each isoenzyme was determined by counting a portion of the eluate for radioactivity and using the remainder of the eluate for quantification of phenylalanine by a previously described isotope-dilution technique using [*naphthalene*-^{14}C]dansyl chloride (Amersham Corp.) and two-dimensional t.l.c. respectively to label and isolate phenylalanine from the other amino acids (Airhart *et al.*, 1979; Everett *et al.*, 1983). The radioactivity in samples of individual MHC isoenzymes obtained from isolated gel bands generally ranged from 75 to 400 c.p.m./0.6 ml aliquot (background was approx. 20 c.p.m.). A notable exception was the level of radioactivity found in the SM1 bands from overloaded ALD muscles at 24 h. Because the amount of SM1 protein and the rate of incorporation of labelled amino acid were so low in these muscles, some were found to contain as little as 22–40 c.p.m. Nevertheless, the results were remarkably consistent within this group, and numerous repetitions of the experiment, including the addition of extra animals, were conducted. Approx. 92% of the total radioactivity was found in phenylalanine, with the remainder in tyrosine, as analysed by two-dimensional chromatographic separation of [^{14}C]dansyl-amino acids.

Total SDS-soluble protein extracts were prepared as previously described (Gregory *et al.*, 1987*a*), with the exception of heating the precipitated proteins at 85 °C for 30 min to release any contaminating precursor [^{3}H]phenylalanyl-tRNA of high S.A. The dried protein was hydrolysed and treated as described above for MHC for the determination of [^{3}H]phenylalanine S.A.

Serial plasma samples and the infusate were analysed for [^{3}H]phenylalanine S.A. by allowing the amino acids to react directly with [^{14}C]dansyl chloride after removal of plasma proteins by precipitation with ethanol.

Fractional synthesis rates (k_s) for total protein and MHC isoenzymes were calculated as follows:

$$k_s\ (\%/\text{day}) = \frac{\text{S.A. of phenylalanine in protein (d.p.m./pmol)} \times 100}{\text{S.A. of phenylalanine in plasma (d.p.m./pmol)} \times \text{time}}$$

where 'time' refers to the duration of the measurement period in days, which in each case included the time taken for dissection of the muscle. Since the plasma [^{3}H]phenylalanine S.A. rapidly reaches a value close to that of the infusate and falls linearly during the course of the measurement period (Garlick *et al.*, 1980; Fig. 1 below), we used the average S.A. during the overall measurement period in the above calculation.

Protein, MHC and actin content

Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The proportions of MHC and actin in total SDS-soluble protein extracts were determined by densitometric scanning of SDS/PAGE gels of total proteins, followed by integration of the area under the peaks. Total MHC and actin were expressed as percentages of the total area.

Translation *in vitro*

Translation *in vitro* of polyribosomes isolated from a separate group of control and overloaded ALD and posterior latissimus dorsi (PLD) muscles was carried out as previously described (Gagnon *et al.*, 1985). Aliquots of polyribosomes containing 0.08 A_{260} unit were added to 15 μl of nuclease-treated rabbit reticulocyte system (Amersham Corp.) containing 50 μCi of L-[^{35}S]methionine (S.A. 1220 Ci/mmol). The potassium concentration was adjusted to 166 mM using the acetate salt. The reaction mixture (30 μl) was incubated for 60 min at 32 °C and terminated by the addition of pancreatic ribonuclease A followed by a 10 min incubation at 37 °C. Radioactivity incorporated into protein was quantified by the method of Mans & Novelli (1961), a 5 μl aliquot being used. Qualitative analysis of the products of translation was carried out by gradient SDS/PAGE (5–15%; Laemmli, 1970) followed by fluorography (Gagnon *et al.*, 1985).

Analysis of mRNA

Total RNA was extracted by using guanidinium thiocyanate and CsCl (Glisin *et al.*, 1974; Chirgwin *et al.*, 1979) from control and overloaded ALD muscles of a separate group of five animals for nuclease-protection analyses and another group of 15 animals for total RNA analysis.

The relative proportions of SM1 and SM2 mRNAs were determined by nuclease-protection analysis. Details of the characterization of the SM1-specific MHC mRNA probe are available from D.A.E. on request. *Bst*N1–*Hin*dIII fragment was isolated from Bluescrybe M13+ (Stratagene) and end-labelled by filling in the 3′ recessed ends with reverse transcriptase (avian myeloblastosis virus; Dr. J. Beard, Life Sciences Inc., St. Petersburg, FL, U.S.A.) in the presence of [α-^{32}P]dATP (Amersham Corp.; Wahl *et al.*, 1979). This fragment contains 213 bp of myosin-coding sequences and 47 bp of the vector. After electrophoretic strand separation of the labelled fragment (Maxam & Gilbert, 1980), the slower-migrating strand complementary to the MHC mRNA was excised from the gel and eluted overnight at 37 °C. Hybridization was carried out at 65 °C in sealed capillary tubes with 0.6 M-NaCl/0.015 M-Pipes (pH 6.4)/1.5 mM-EDTA for 18–20 h (Orkin & Goff, 1981) and 30000 c.p.m./g of probe. Digestion of the hybrids with 470 and 675 units of mung-bean (*Phaseolus aureus*) nuclease (Pharmacia) was carried out in 0.2 M-NaCl/0.05 M-sodium acetate (pH 4.6)/0.001 M-$ZnSO_4$ for 4 h at 37 °C, followed by analysis on 8.3 M-urea/5%-(w/v)-polyacrylamide sequencing gels, autoradiography and liquid-scintillation spectrometry of excised fragments.

The percentage of mRNA in total RNA extracts was determined by hybridization of RNA with a [^{32}P]poly(U)probe. Equal amounts (determined by A_{260}) of control and overloaded RNA were slot-blotted (Schleicher and Schuell, Keene, NH, U.S.A.) on to nitrocellulose (Maniatis *et al.*, 1982). Hybridization and washing conditions were as described by Harley (1987). Quantification of the proportion of mRNA was carried out by densitometric scanning of autoradiograms.

Statistical analyses

Differences between overloaded and contralateral control muscles, or between two proteins in the same muscle, were evaluated with a paired *t* test (Snedecor & Cochran, 1968). A value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Muscle growth and isomyosin transition in overloaded ALD in young growing chickens

After the attachment of a lead weight of approx. 10% of the animal's body weight, the ALD muscle underwent a rapid increase in wet weight (Table 1). By 72 h, overloaded muscles were 50% heavier than contralateral controls. Thus the speed and extent of ALD muscle growth were high and substantial during the period in which protein-synthesis measurements were made. The weight of the fast PLD muscle was not significantly increased over controls after 72 h of overload (244 ± 10 mg as against 218 ± 11 mg for controls; $n = 5$). Body weights of animals in the 24 h group (442 ± 20 g; $n = 8$) were greater ($P < 0.05$) than those of the 48 h (367 ± 12 g; $n = 5$) and 72 h (398 ± 14 g; $n = 6$) group, probably owing to lack of precise weight matching at the beginning of the experiment. However, all experimental data were based on comparisons with contralateral control muscles for internal control.

At 24 h after overloading the ALD muscle, the relative amount of SM1 was unchanged (Table 1). By 48 h, however, the level of SM1 was significantly reduced, and at 72 h it was decreased from contralateral control values by nearly 50%. These data are consistent with those obtained previously (Kennedy *et al.*, 1986).

Precursor equilibration

The S.A. of [^{3}H]phenylalanine in plasma reached a level that was approx. 97% of that of the infusate within the first 2 min and fell linearly throughout the course of the experiment to about 80% of the initial value at the end of 40 min (Fig. 1). The S.A. of [^{3}H]phenylalanine in the intracellular free pool was found to be equal to that of the final plasma sample (results not shown). The rapid equilibration and linear fall in S.A. are characteristic of the 'flooding-dose' technique (Garlick *et al.*, 1980), which is believed to flood all intracellular and extracellular compartments with radiolabelled amino acid of uniform S.A., including the precursor [^{3}H]phenylalanyl-tRNA. The latter assumption, however, has not been tested. If the S.A. of [^{3}H]phenylalanyl-tRNA did not reach the plasma values, the synthesis rates we report here would

Table 1. Muscle hypertrophy, protein content (*a*) and myosin isoenzyme transitions (*b*) in control (C) and overloaded (OV) ALD muscles

Results are means ± S.E.M., with number of muscles studied in parentheses. Percentage of MHC and percentage of actin refer to the percentage of total SDS-soluble protein which is MHC or actin respectively. The percentage of SM1 refers to the percentage of total MHC which is SM1. * $P < 0.05$, significantly different from contralateral control (paired t test).

(*a*)

Time (h)	Muscle weight (mg)		Protein (mg/g)	
	C	OV	C	OV
24	178±12 (8)	242±20 (8)*	122±2 (5)	106±3 (5)*
48	137±11 (5)	214±19 (5)*	127±5 (4)	115±3 (5)*
72	171±7 (6)	254±12 (6)*	125±4 (5)	115±4 (5)*

(*b*)

Time (h)	Percentage of: MHC		Actin		SM1	
	C	OV	C	OV	C	OV
24	23±1 (5)	19±1 (1)	13±1 (5)	11±2 (5)	35±4 (5)	37±3 (5)
48	22±1 (3)	18±1 (3)	16±1 (3)	16±1 (3)	35±1 (5)	27±7 (5)*
72	23±1 (5)	17±1 (5)*	15±1 (5)	15±1 (5)	35±3 (6)	19±2 (6)*

be underestimated. However, since we used the contralateral muscle as the control, the differences between control and overloaded muscles reported here would be the same, unless there were differences between control and overloaded muscles in the rate or the extent of equilibration. Even if such differences existed, the relative rates of SM1 and SM2 synthesis we report would still be correct, assuming similar equilibration parameters among individual fibre types within the ALD. Finally, the relationships among rates of total protein, actin and myosin synthesis we report here would be preserved regardless of precursor equilibration characteristics.

Synthesis of both MHC isoenzymes are initially repressed in overloaded ALD muscle whereas actin and total protein are increased

Table 2 shows the fractional synthesis rates (k_s) of total protein, actin and myosin isoenzymes in control and overloaded ALD muscles. The k_s of total protein was nearly doubled at 24 h and continued to rise at 48 and 72 h. The growth rate (k_g) of the control ALD is approx. 4%/day (Kennedy *et al.*, 1986). Thus the rate of protein synthesis, even in control muscles (14%/day), exceeded the growth rate. The overall protein degradation rate, k_d, can be calculated by using the equation:

$$k_d = k_g - k_s$$

and is about 10%/day in control ALD muscle. Increased degradation clearly plays a role during overload, when k_s is approximately twice k_g (15%/day; Kennedy *et al.*, 1986). Increased degradation has been observed in several models of muscle hypertrophy (Laurent *et al.*, 1978; Goldspink *et al.*, 1983) and may be an important feature of the remodelling which accompanies hypertrophy.

In control ALD muscle the k_s of SM1 was lower than that of SM2 ($P < 0.001$), a difference which probably reflects the gradual loss of SM1 that occurs during normal ALD-muscle development (Kennedy *et al.*, 1986). Surprisingly, the k_s of both myosin isoenzymes was depressed at 24 h, particularly that of SM1, which fell by about 70%. By 48 h, however, the k_s of SM2 was nearly doubled, and at 72 h, the k_s of both SM1 and SM2 was almost three times greater than the control. This delay in myosin synthesis was not a characteristic of all contractile

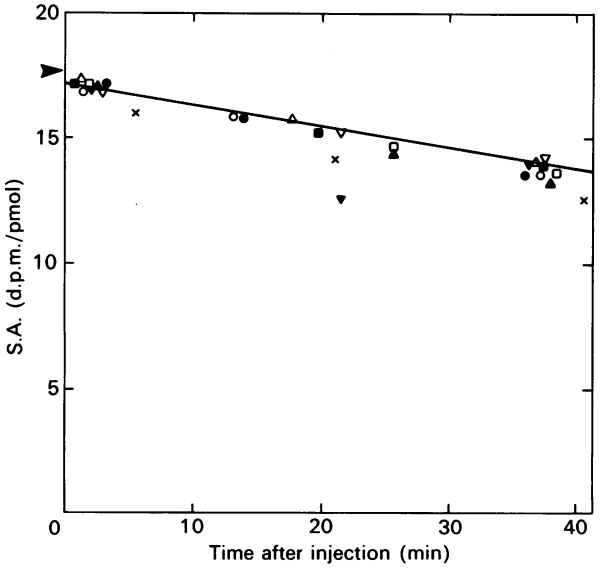

Fig. 1. S.A. of [^{3}H]phenylalanine in plasma

After injection of a 'flooding' dose (150 μmol/100 g body weight) of L-4-[^{3}H]phenylalanine, blood samples were taken at the beginning, middle and end of the period of protein-synthesis measurement, for the determination of the S.A. of the precursor [^{3}H]phenylalanine. Individual symbols represent the three data points for a single animal. The S.A. of the injected [^{3}H]phenylalanine is indicated by the arrowhead.

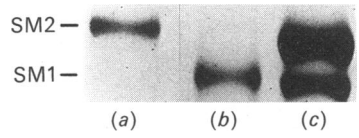

Fig. 2. Assessment of purity of MHC isoenzyme preparations

Individual MHC isoenzymes were isolated by immunoadsorbent chromatography for the measurement of synthesis rates. Each preparation was subsequently analysed by SDS/PAGE, followed by silver staining, to ensure that there was no contamination by the other isoenzyme. A representative SM2 preparation (*a*), SM1 preparation (*b*) and mixture of the SM2 and SM1 preparations (*c*) are shown.

proteins, since the k_s of actin was doubled at 24 h and continued to increase further (Table 1). Thus there was a delay in the stimulation of myosin synthesis and an independent response to two major contractile proteins to the overload stimulus.

The relationship between actin and MHC synthesis rates changed with overload (Table 2). Whereas the k_s for actin was significantly less than that of MHC in control ALD muscle ($P < 0.01$), this relationship was reversed at 24 h, when the actin k_s was severalfold greater than that of MHC and returned to near normal by 72 h. The possible implications of MHC lagging behind actin expression are discussed below.

Table 2. Fractional synthesis rates (k_s) *in vivo* of total SDS-soluble protein, actin and MHC isoenzymes in control (C) and overloaded (OV) muscles

Values are expressed as mean ± S.E.M., with the number of muscles studied in parentheses. *Significantly different from contralateral control, paired *t* test, $P < 0.05$. The separation of SM1 and SM2 isomyosin is shown in Fig. 2.

(*a*) ALD muscle

	Time (h)	k_s (%/day) C	k_s (%/day) OV
Total protein	24	14.0±1.1 (8)	24.7±1.6 (8)*
	48	14.3±0.8 (5)	31.3±1.8 (5)*
	72	14.7±0.7 (6)	34.4±1.2 (6)*
SM1	24	7.4±0.7 (7)	2.1±0.5 (6)*
	48	6.0±0.7 (4)	10.4±1.8 (6)
	72	6.3±0.6 (5)	19.3±2.4 (5)*
SM2	24	11.9±1.6 (8)	8.4±1.3 (8)*
	48	11.8±0.5 (5)	23.7±2.4 (5)*
	72	11.1±1.3 (6)	29.8±2.4 (6)*
Actin	24	7.6±0.9 (6)	18.5±3.7 (5)*
	48	7.1±0.3 (5)	23.1±1.0 (5)*
	72	8.9±1.7 (4)	22.0±2.5 (5)*

(*b*) PLD muscle

	Time (h)	k_s (%/day) C	k_s (%/day) OV
Total protein	24	6.2±0.6 (5)	9.1±0.4 (5)*
	48	5.7±0.7 (5)	13.1±0.7 (5)*
	72	6.6±0.5 (5)	11.9±0.9 (5)*
MHC	24	4.4±0.4 (5)	5.2±0.7 (5)
	48	3.9±0.5 (5)	8.9±0.7 (5)*
	72	4.6±0.6 (5)	8.2±0.4 (5)*

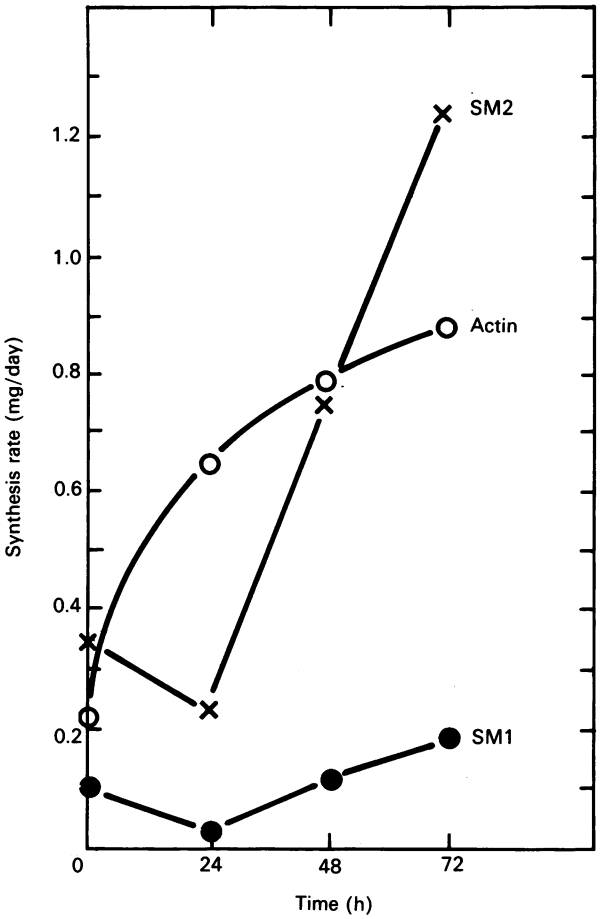

Fig. 3. Synthesis rates of actin and myosin heavy chain isoenzymes in the ALD muscle

Each point in the Figure was obtained by multiplying fractional synthesis rates by the amount of each protein present. An estimate of statistical error is not given because values were calculated from several group means listed in Tables 1 and 2.

Since the overloaded ALD muscle increased in weight and protein content and the proportion of myosin isoenzymes changed, we also expressed the synthesis rates in mg of protein synthesized/day by multiplying the fractional synthesis rate by the amount of each myosin isoenzyme present at each time point (Fig. 3). The difference between the amount of SM1 and SM2 synthesized was much greater than implied by the fractional synthesis rates. There was a fivefold increase in the amount of SM2 synthesized, whereas the amount of SM1 synthesized remained near control levels. Thus the syntheses of individual myosin isoenzymes were independently controlled.

Because of the unexpected decrease in myosin synthesis *in vivo* that we found in the overloaded ALD muscle at 24 h, we sought an alternative method as well as an independent means of measuring synthesis rates. First, to determine whether the method of isolation of MHC affected the observed synthesis rates, we measured the k_s

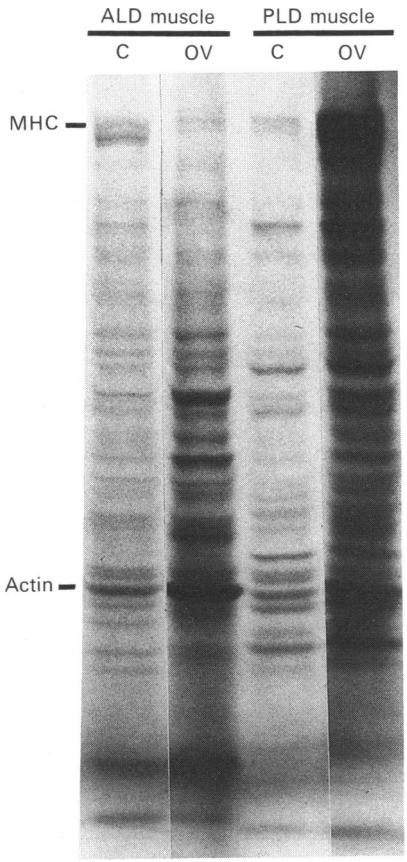

Fig. 4. Translation *in vitro* of polyribosomes isolated from ALD and PLD muscles after 24 h of overload

An identical amount of polyribosomes (A_{260} 0.08) from each muscle was translated *in vitro* in the presence of L-[^{35}S]methionine, and the products of translation analysed by SDS/gradient (5–15%, w/v) PAGE, followed by fluorography. C, control; OV, overloaded.

of total MHC in control and overloaded ALD. The data obtained by this alternative method (preparative SDS/PAGE) were in good agreement with those data obtained by immunoadsorbent chromatography (e.g. at 24 h the k_s of total MHC was 11.5±1.8%/day in control ALD muscle and 7.8±2.4%/day in overloaded ALD muscle). Secondly, polyribosomes isolated from control and overloaded muscles at 24, 48 and 72 h were translated *in vitro*. At 24 h, the overall synthesis of proteins by the same amount of polyribosomes, measured by [^{35}S]methionine incorporation, was greater ($P < 0.05$) in overloaded ALD muscle (8081±1224 c.p.m.; $n = 5$; Fig. 4) than in the control (6133±418 c.p.m.; $n = 5$). A similar increase was observed at 48 and 72 h (results not shown). Although actin synthesis was increased in both the ALD and PLD muscles, MHC synthesis was depressed in the ALD muscle and increased in the PLD muscle, results consistent with those obtained *in vivo* (see below and Table 2). The faint MHC band in the ALD polyribosome translation (Fig. 4) cannot be due to failure of the '*in vitro*' translation system to complete the translation of large mRNA transcripts, because the MHC mRNA is adequately translated in the control ALD muscle and in both control and overloaded PLD muscle samples. Thus the delay in MHC synthesis observed *in vivo* appears to be real.

SM1 isoenzyme is selectively degraded in overloaded ALD

The observed loss of SM1 from overloaded ALD muscle could be due to decreased SM1 synthesis, selective degradation of SM1 or both. To determine whether the decrease in SM1 synthesis could fully account for the loss of SM1 from overloaded ALD muscle, we predicted how much SM1 would be lost due to the decrease in SM1 synthesis we observed, and compared this with the amount of SM1 actually present in the ALD muscle after 72 h of overload (calculated from the muscle weight, protein concentration, percentage of MHC and percentage of SM1; Table 2). Theoretically, if SM1 synthesis were completely shut down, with no change in the rate of degradation, the predicted rate of loss for the existing pool of SM1 would be approx. 7%/day, or about 20% in 72 h. At 72 h, the control ALD was observed to contain 1.7 mg of SM1, whereas the overloaded ALD contained only 0.9 mg of SM1, a loss of almost half the

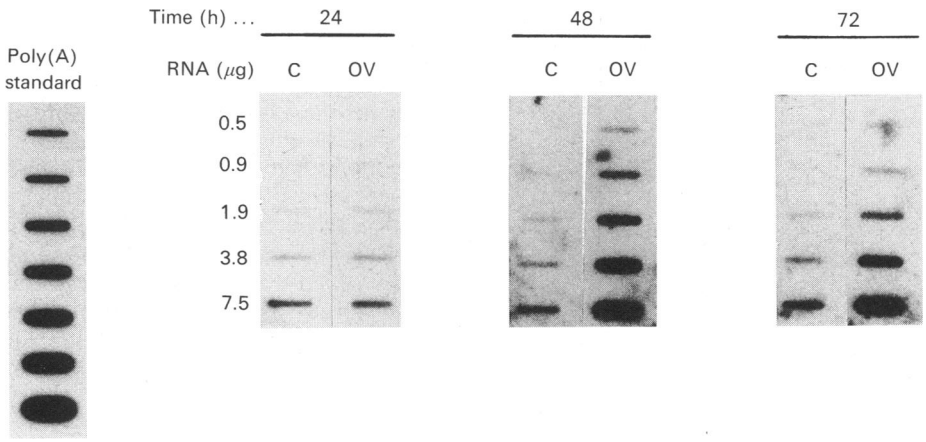

Fig. 5. Analysis of total poly(A)-containing mRNA

Equal amounts of total RNA isolated from control (C) and overloaded (OV) ALD muscle were blotted on to nitrocellulose and hybridized with [^{32}P]poly(U). Representative autoradiograms from 24, 48 and 72 h groups are shown. The linearity of this assay is demonstrated in the poly(A) standard on the left.

SM1 originally present. Since the assumptions we made in predicting the amount of SM1 loss were conservative [the synthesis rate of SM1 was not completely shut down at 24 h (Table 2) and returned to near control values by 48 h], the difference between the predicted and observed values for SM1 loss is almost certainly greater than this. Thus the only way a loss of this magnitude could have occurred is by increased specific degradation of the SM1 isoenzyme. Since the SM1 and SM2 isoenzymes are largely contained in discrete fibre types in 5-week-old chickens (Kennedy *et al.*, 1986), it is likely this increased degradation was confined to a particular subset of ALD-muscle fibres. During overload, those SM1-containing fibres are not degraded and replaced (Kennedy *et al.*, 1988) but switch to the expression of SM2 (Kennedy *et al.*, 1986), suggesting that the increased degradation was specific for SM1 rather than a generalized catabolic state. Thus whereas the gradual developmental replacement of SM1 by SM2 in control ALD muscle appears to occur by differential synthesis (compare k_s of SM1 and SM2 in control ALD; Table 2), the accelerated replacement of SM2 by SM1 during overload hypertrophy may require in addition a selective increase in SM1 degradation.

Differential response of actin and MHC synthesis leads to a disproportion in their contents

The protein concentration in the ALD muscle was significantly less than that in contralateral controls at all three time points (Table 1), probably as a result of the increased water content of overloaded ALD muscle ($80.8 \pm 0.3\%$ as against $77.4 \pm 0.2\%$ for controls; $n = 16$; $P < 0.001$) and the depression of myosin synthesis at 24 h. There was no significant difference between the protein concentration in the control and that in the overloaded PLD muscle [at 24 h, the control PLD-muscle value was 185 ± 8 mg/g and that for the overloaded PLD muscle was 187 ± 13 mg/g ($n = 5$)]. The percentage of the total protein composed of MHC was decreased in overloaded ALD muscle at 72 h (Table 1). The percentage actin content, however, was not significantly changed. Thus the non-co-ordination of actin and myosin synthesis in response to the overload stimulus resulted in a decreased MHC content and an altered ratio of actin to MHC content (control/overload 0.67:0.90 at 72 h). Whether there are any functional consequences of a disproportion of actin and myosin contents is not clear. However, our measurements did not distinguish between the various isoforms of muscle-specific and non-muscle actins. It is conceivable that some of the actin synthesized was non-muscle actin, since there is a substantial increase in the amount of non-myofibrillar material in the ALD muscle during the first few days of overload (Kennedy *et al.*, 1988). Alternatively, it is known that normal myofibrillar integrity is temporarily lost in overloaded ALD muscle owing to overstretching of the sarcomeres beyond filament overlap (Kennedy *et al.*, 1988). Thus the excess actin synthesized may be muscle-specific actin that remained in a free pool of unassembled contractile proteins. Similarly to results of our study carried out at the protein level, the accumulation of mRNA coding for α-skeletal actin also precedes the increase in the abundance of β-MHC message, as has been shown during the early phase of cardiac enlargement induced by pressure overload (Schiaffino *et al.*, 1989).

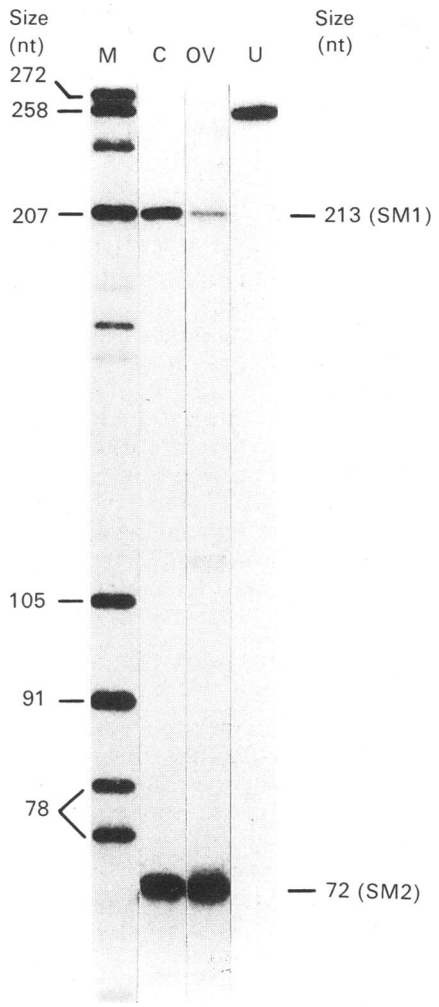

Fig. 6. Nuclease protection analysis of control and overloaded ALD muscle RNA

Total RNA was extracted from contralateral control (C) and overloaded (OV) ALD muscles at 72 h, hybridized with the SM1-mRNA-specific cDNA probe (isolated by means of endonucleases *Bst*N1–*Hind*III from subclone 15/2-S33) and digested with mung-bean nuclease. The 213-nt fully protected fragment represents the SM1 mRNA and the 72-nt partially protected fragment represents the SM2 mRNA. M, markers (*Sau*3A-digested PBR322); U, undigested probe.

There is no delay in the stimulation of MHC synthesis in the fast PLD muscle

The k_s of total protein and of total MHC in the fast PLD muscle were lower at all time points in both control and overloaded muscles than those values observed at corresponding time points in the slow ALD muscle (Table 2), consistent with a previous report (Laurent *et al.*, 1978). There was no depression of the myosin synthesis rate after 24 h of overload as was observed in the ALD muscle. Fractional synthesis rates were doubled at 48 and 72 h for both total protein and MHC in the PLD muscle, despite the lack of significant increase in muscle weight in the PLD muscle (see above). Thus, in the fast PLD muscle, the increase in protein synthesis rates is rapid and substantial, despite a lower rate of

muscle hypertrophy (21% increase in muscle weight at 7 days; R. Zak, unpublished work).

Total mRNA is increased at 48 and 72 h, but not at 24 h, despite the increase in total protein synthesis at 24 h

To obtain an insight into the regulatory mechanisms of the observed changes in protein synthesis, we measured the percentage of total RNA which was poly(A) mRNA. Fig. 5 shows the results of RNA blot hybridization of total RNA from control and overloaded ALD muscle with a [^{32}P]poly(U) probe, which hybridizes to the poly(A) tail of mRNA molecules. At 24 h, there was no change in the percentage of mRNA per absorbance unit of total RNA [control, 35 ± 9; 24 h overload, 40 ± 6 (arbitrary absorbance integration units); (not significant)]. However, there was a 2.5-fold increase in the proportion of mRNA at 48 h (84 ± 13; $P < 0.001$) and a 3-fold increase at 72 h (108 ± 13; $P < 0.001$). This suggests that, at 24 h, different regulatory mechanisms are involved than at 48 and 72 h. Thus translational control mechanisms may be important in the initial phase of hypertrophy.

Relative amount of SM1 mRNA in the ALD decreases during overload

We analysed the relative proportion of mRNAs coding for SM1 and SM2 MHCs after 3 days of overload, using the method of nuclease-protection analysis, to determine whether the decrease in SM1 protein was reflected in a similar decrease in its corresponding mRNA. Full protection of the cDNA probe resulted in a 213-nucleotide (nt) fragment representing the SM1 MHC mRNA (Fig. 6). This fragment can be distinguished from any undigested probe (260 nt), which contains an extra 47 nt of vector sequences. Because of the sequence conservation between portions of the two MHC isozymes, a smaller partially protected fragment (72 nt) was produced which represents the heterologous SM2 MHC mRNA. After 3 days of overload, the percentage of SM1 mRNA was only 18% of the total MHC mRNA, compared with 30% in the contralateral control ALD muscle. Thus the SM1 → SM2 transition that occurred during ALD-muscle hypertrophy was accompanied by a decrease in the relative amount of SM1 mRNA.

In conclusion, we have found that the myosin isoenzyme transition that occurs during overload hypertrophy of the ALD is accompanied by dramatic alterations in the synthesis and degradation of individual myosin isoenzymes. Moreover, we found that the regulatory mechanisms are gene-specific and differ in the initial phase of and later stages of hypertrophy.

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