Gene expression in skeletal muscle in response to stretch and force generation

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Goldspink, Geoffrey, Andrew Scutt, Paul T. Loughna, Dominic J. Wells, Thomas Jaenicke, and Gerald F. Gerlach. Gene expression in skeletal muscle in response to stretch and force generation. *Am. J. Physiol.* 262 (*Regulatory Integrative Comp. Physiol.* 31): R356–R363, 1992.—Striated muscle is a tissue in which gene expression is influenced to a large extent by mechanical signals. This includes the regulation of gene expression-associated muscle fiber phenotype determination, which depends on which protein isoform genes are transcribed, as well as muscle fiber mass accretion, which appears to involve some translational regulation. Although muscle synthesizes a set of highly specialized proteins it has a remarkable ability to adapt by expressing different isoforms of the same protein so that it acquires the appropriate contractile characteristics. Our work has focused on the myosin heavy chain (HC) genes as these encode the myosin cross bridge, which is responsible for muscle intrinsic velocity of contraction and economy of force development. RNA analyses after cast immobilization of the limb with the muscle in the lengthened or shortened position and/or with electrical stimulation were used to determine the effects of altered mechanical signals on gene transcription. When the soleus muscle was immobilized in the shortened position in the young animal it did not fully differentiate into a slow postural-type muscle. Even in the adult, the soleus muscle if deprived of stretch and contractile activity switches back to transcribing the fast myosin HC gene. The converse was true when the fast rabbit tibialis anterior was subjected to immobilization in the lengthened position and/or electrical stimulation. Both stretch alone and stimulation alone caused repression of the fast type and activation of the slow myosin genes. The reprogramming of the fast muscle was more complete when the stretch was combined with stimulation. When subjected to stretch and mechanical overload skeletal muscle apparently adapts to a more postural type of role by expressing the slow isoform genes as well as higher levels of mitochondrial genes. As far as muscle mass accretion is concerned, stretch combined with force generation caused the tibialis anterior in the adult rabbit to increase in mass by 30% within 4 days. This was associated with an increase in total muscle RNA of 250%, which reflects a large increase in ribosomes available to translate whatever message is available. The possible link between the mechanical signal and the activation or repression of certain muscle genes is discussed.

stimulation; hypertrophy; myosin genes

MAMMALIAN SKELETAL MUSCLES consist of populations of slow-contracting oxidative fibers that are adapted for slow repetitive or postural-type contractile activity and fast-contracting fibers that are recruited for fast phasic movements. The muscle fiber types differ phenotypically in that they express different subsets of myofibrillar isoform genes as well as different types and levels of metabolic enzymes. The inherent ability of skeletal muscle to adapt to mechanical signals is related to its ability to switch on or switch off different isoform genes and to alter the general levels of expression of different subsets of genes. The fact that there are several myosin heavy chain (HC) isoforms means that a muscle fiber can alter its contractile properties by rebuilding its myofibrils using a myosin HC with a slow or fast cross-bridge cycling rate. The intrinsic velocity of contraction (V_{max}) of muscle fibers has been related to the specific activity of their myosin ATPase (2). Myosin is a double molecule that consists of two HCs each of about 200,000 Da. The actin attachment site and the ATPase site are located in the S_1 region (head of the myosin cross bridge) of the HCs. Associated with the S_1 fragment are smaller polypeptides or light chains that are believed to modulate the cross-bridge ATPase activity (16). We have focused our attention on the myosin HC genes because of the strong correlation between the V_{max} of the muscle fibers and their fast and slow myosin HC content (43).

The isoforms of myosin HC have been shown to be the products of a multigene family, and their expression is tightly regulated in a stage-specific and tissue-specific manner (6, 32, 38, 54–56). Phenotypic expression of muscle genes is known to be influenced by thyroid hormone (21, 28) and altered patterns of innervation (41). However, the influence of physical activity at the gene level was unclear; therefore we have studied changes in gene expression of the fast and slow genes in response to the two main mechanical stimuli: stretch and force generation.

IMPORTANCE OF STRETCH ON EXPRESSION OF SLOW TYPE GENES

The soleus muscle of mammals is a postural muscle that consists of a mixture of slow oxidative (type I) and fast oxidative (type IIa) fibers with no fast glycolytic (type IIb) fibers. The type I fibers strongly express the cardiac β-myosin (slow), which seems to be a characteristic only of slow postural muscle fibers. To investigate the role of early activity on slow muscle fiber-phenotype development in the soleus, the limbs of 3- to 7-day-old mice and rats were immobilized for 2–3 wk with the foot plantar flexed (soleus in the shortened position and inactive). Initially adhesive tape and splints were used, switching to plaster casts at ~2 wk of age. The level of expression of slow myosin in the rat soleus was assesesd by in situ hybridization using cRNA probes to the 3′ untranslated region of the rat β-cardiac gene (Fig. 1*A*). This indicated that, although this gene is expressed in the embryonic and neonatal muscle (37, 54), lack of activity reduced expression of the β-myosin mRNA.

This paper was presented at The First International Biomechanics Congress held in San Diego, CA, September 3, 1990.

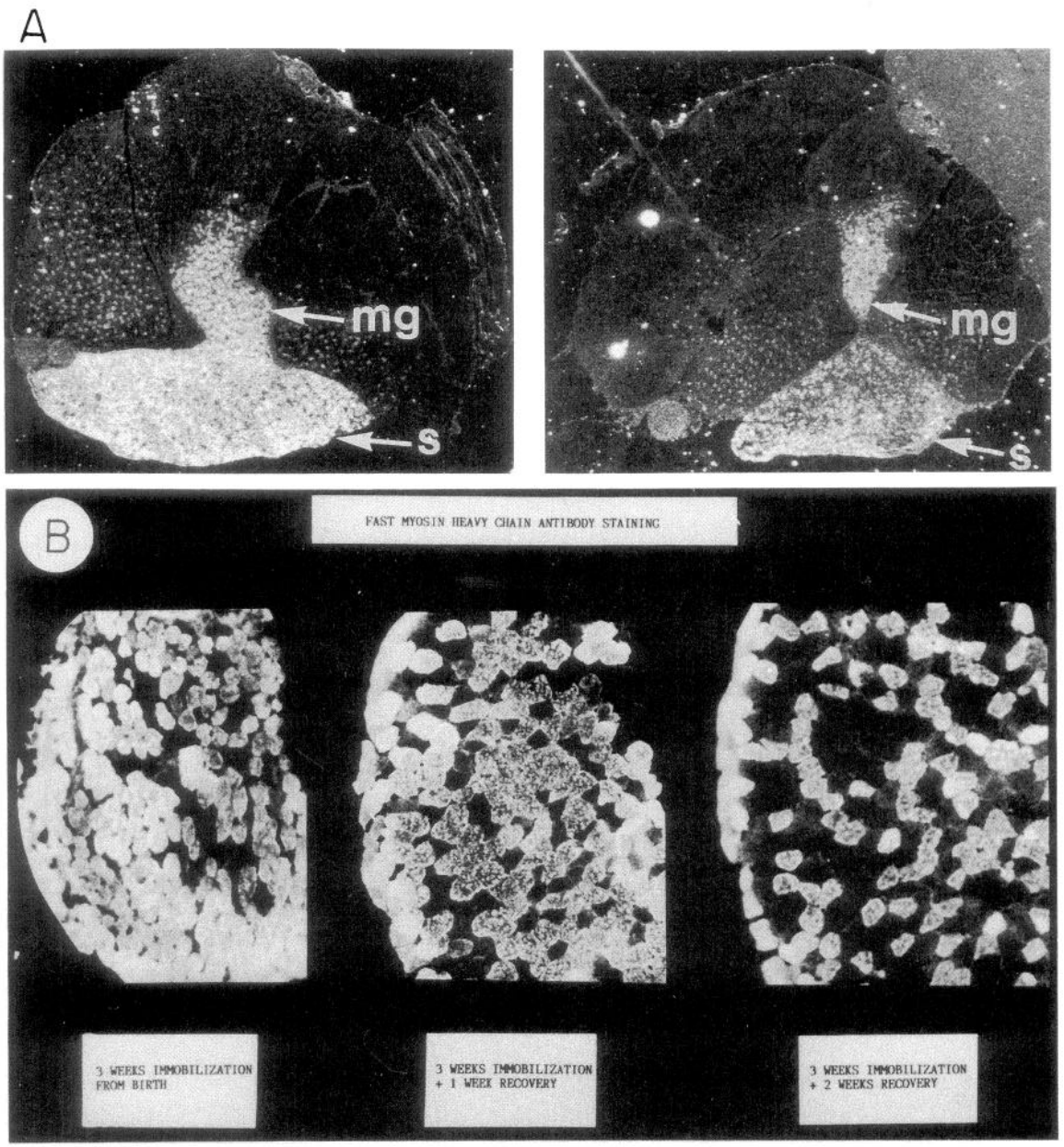

Fig. 1. *A*: transverse sections of the young rat soleus, plantaris, and gastrocnemius muscle group subjected to in situ hybridization with a pMHC 5-derived 3′ untranslated rat β-myosin heavy chain (HC) ^{35}S-labeled cRNA. *Left*: control leg; *right*: experimental leg after 2 wk immobilization with tarsal joint plantar flexed. As seen from these autoradiographs there is a reduction in β-slow myosin HC mRNA in the immobilized soleus (s) and the incompletely immobilized medial head of the gastrocnemius (mg) compared with the control muscles. *B*: transverse sections of the mouse soleus muscle stained with a fluorescently labeled fast myosin heavy chain antibody donated by Dr. Wendy Brown. *Left to right*: after 3 wk immobilization in the shortened position commencing shortly after birth, 3 wk immobilization plus 1 wk recovery, and 3 wk immobilization plus 2 wk recovery. All the fibers in the young muscle immobilized in the shortened position for fast myosin (white). After removal of the plaster cast, the fast IIb gene starts to be suppressed and the establishment of the characteristic checkerboard appearance is apparent by 2 wk recovery, when the animal has fully regained the postural function of the soleus muscle.

After the cast was removed and the muscle used in locomotion, i.e., subjected to stretch and force generation, then it strongly expressed β-myosin mRNA, and consequently the muscle slow isomyosin content became comparable to the control animals. Incomplete suppression of the β-myosin HC mRNA in immobilized muscles in very young animals may be due to the endogenous stretch generated by the rapid skeletal growth that is occurring during the early postnatal period. In the mouse, frozen sections stained for myosin ATPase, succinic dehydrogenase, and antibodies to slow myosin showed that phenotypic differentiation of the fibers did not occur unless the muscle was used for its postural function (Fig. 1*B*). Other subsets of genes are apparently regulated in a similar manner because electrophoretic separation of the isoforms of lactic dehydrogenase (LDH) demonstrated that the newborn mouse soleus had a banding pattern characteristic of fast skeletal muscle and that

this was retained if the muscle was immobilized in the shortened position (15). The slow heart-type LDH isoform pattern was attained after ~3 wk of recovery from immobilization. These experiments indicate that fiber phenotype is dependent to a large extent on mechanical activity and less rigidly predetermined than appears to be the case for avian muscle (34). The slow cardiac β-myosin HC is known to be transcribed as a minor component in fetal skeletal muscle (30), and this occurs mainly in the primary fibers (37), which could be taken as meaning that these are committed to become the slow postural type I fibers. However, it might be argued that the primary fibers are the first to develop and engage in contractile activity and to be subjected to stretch by the considerable lengthening of the bones in the developing fetus, and therefore they would be expected to express slow myosin. Evidence for the absence of commitment to a distinct slow myoblast lineage fiber type in mammalian skeletal muscle was recently presented by Travis et al. (50), who analyzed myoblast clones derived from different stages of human development and found that during the first trimester 100% expressed slow myosin HC. Midgestation, only 3% of myoblasts expressed the slow and towards the end of gestation 50% expressed the slow myosin HC. They concluded that it is the extrinsic factors prevailing at the time that induce or activate the expression of the slow gene.

The muscles in young mammals just after birth show considerable plasticity. In experiments where the muscles were overloaded by tenotomy of the synergistic muscle, there was an increase in the percentage of slow type I fibers and a considerable increase in intermediate or type IIc fibers (52). Ballistic action (jumping for food) delayed this fast-to-slow conversion in the soleus (53). Previous histochemical studies also reported that there is normally a progressive increase in the percentage of slow fibers in the soleus (25, 44), although the opposite shift occurred in fast muscles. Similar findings have recently been reported (27) using an enzyme-linked immunosorbent assay antibody method that shows that the slow myosin HC expression is increased in the soleus in the young rat and is increased further by overload. Certainly, in older muscles, overload induced by tenotomy of their synergistic muscles is known to result in the transformation of fast to slow (17, 20), which appears to be due to a suppression of type IIb myosin HC genes (36) as well as the activation of the slow genes. Further data that stretch and activity determine which isomyosin gene is transcribed were obtained in experiments in which S_1 nuclease mapping with rat gene-specific probes was used to analyze the changes in the species of mRNA after immobilization in the stretched and nonstretched positions (27). One of the most interesting findings was that the soleus muscle, which does not normally express the fast glycolytic (type IIb) myosin HC gene, commenced to transcribe this gene at a high level when the muscle was immobilized to the shortened position. Appreciable levels of the fast myosin HC mRNA were detected after only 2 days (Fig. 2). It seems therefore that the fast type IIb gene is the default gene and that this is transcribed unless the muscle fibers are subjected to stretch and/or force generation. This has been shown at the histochemical level (23) as well as the gene expression level (15). Gene constructs that include a type I slow β-myosin HC promoter spliced to a luciferase reporter sequence have been introduced into the rat soleus by injection using the technique of Wolff et al. (62). These constructs are not transcribed if the soleus muscle is denervated (49). This again indicates that it may be active tension that is linked to switching on the slow type I myosin HC gene. The expression of the slow gene subset may be a function of the length of time for which a fiber is activated. As can be seen by its electromyogram (EMG) profile, the soleus muscle in the rat is active all the time the animal is standing, while a fast muscle such as the extensor digitorum longus is only active 5% of the time (18). Although there is evidence that the β-myosin HC gene is expressed before birth (30, 37, 51), it seems that early postnatal activity is important in the establishment of the slow muscle fiber phenotype. However, because motoneurons control the activity of the developing muscle fibers, this could be interpreted as predetermination. Certainly, the pattern formation that occurs in the central nervous system and the differentiation of the motoneurons into fast type or slow type will then determine the fate of the fibers in the motor units they innervate. Innervation is important, but again it must be said that it is probably the kind of activity they evoke rather than the type of innervation per se that induces the slow phenotype.

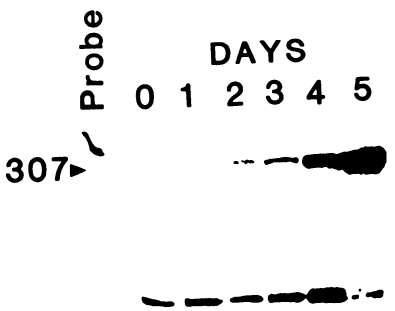

Fig. 2. Expression of the fat glycolytic (type IIb) myosin heavy chain gene in the rat soleus subjected to immobilization in the shortened position for up to 5 days. The probe used was a single-stranded 307 nucleotide 3′ end of pMHC 62. S1 nuclease digestion was carried out following hybridization to remove any single-stranded RNA or DNA. [From Loughna et al. (29).]

RAPID GENE SWITCHING CAUSED BY STRETCH COMBINED WITH ELECTRICAL STIMULATION

To investigate the role of force generation, the fast tibialis anterior (TA) muscle in the adult Netherland dwarf rabbit was stimulated using Teflon-covered stainless steel electrode wires implanted in the popliteal fossa (60). The electrode wires were externalized at the back of the neck and attached to a miniature stimulation circuit that was held in position by a small saddle. Several designs of stimulator circuit were used, including some that gave low-frequency continuous trains and some that gave higher-frequency intermittent trains. The 30- and 60-Hz intermittent stimulation circuits were designed to give the same number of pulses per minute as 2- and 10-Hz continuous stimulation circuits, and in this way the hypothesis that the number of pulses is important could

be tested. In all cases the pulses were biphasic and the pulse voltage was adjustable up to a maximum of 3 V. Staining of frozen sections with monoclonal antibodies specific for slow skeletal myosin HCs revealed a marked increase in the number of fibers expressing slow myosin after only 4 days of stretch and 30 Hz of stimulation (Fig. 3). Measurements of the specific ATPase of myofibrils prepared from rabbit TA muscles also indicated that after 7 days there was a significant slowing of the stretched (17%) and of the stimulated (30 Hz) and stretched (30%) muscles compared with their controls (14).

To study the switches in gene expression, Northern blots were carried out using total RNA extracted from muscles that had been stretched and/or electrically stimulated. Hybridization was carried out using a cDNA fragment for the slow β-type myosin HC that was originally cloned and characterized in the laboratories of Zak, Rabinowitz, and Umeda (46, 51) or a 350-base-pairs fragment specific for the fast type IIb myosin HC cut out from the fragment cloned in Wittinghofer's laboratory (31). A fast myosin light chain cDNA that includes coding sequences for myosin light chains 1 and 3 was also obtained from Dr. Wittinghofer. Stretch combined with electrical stimulation was found to induce very rapid hypertrophy of TA muscle in the adult animal. Increases of up to 30% wet wt were recorded in a period as short as 4 days. Both stretch and force generation are major factors in activating protein synthesis, and the combination of these stimuli apparently has a synergistic effect. Associated with this very significant increase in muscle size, there was a marked increase (up to 250%) in RNA content of the muscles that was found to peak after 2 days from commencement of stretch. Northern analyses showed that there was a marked switch in the levels of mRNA of the slow myosin HC genes (Fig. 4*A*). In response to both stretch and electrical stimulation, the fast TA becomes reprogrammed to transcribe slow myosin HC and to repress the expression of the fast myosin HC gene within only 4 days. Using in situ hydridization, Dix and Eisenberg (9) showed the same increase in the slow myosin HC mRNA in the rat TA muscle after 4 days of stretch and observed a greater accumulation of the message at the ends of the fibers. This is in accord with our finding that the newly synthesized slow myosin HC is more apparent at the ends of the fibers where new sarcomeres are being added in series in response to stretch (59).

The identity of the slow myosin gene that is expressed in the tibialis anterior is in some doubt since high stringency and long exposure Northern blots using the more specific β-myosin probe for the (*Sac*1 fragment of pMHCβ 174) 3′ region indicated that it is not the β-cardiac gene that is induced. Indeed, although there was some hybridization, the β-cardiac was if anything also repressed during stretch and stimulation (Fig. 4*B*). We therefore postulate that there is another skeletal slow myosin HC gene that has yet to be cloned and characterized. The β-cardiac gene is undoubtedly expressed in the soleus muscle (26), and this is no doubt why it is thought to be the only slow myosin HC gene expressed in skeletal muscle. There is good evidence for a separate fast glycolytic/oxidative IIa gene as distinct from the fast glycolytic IIb gene (22, 58). Recent electrophoretic and peptide mapping data suggest that there are also two skeletal muscle type IIa isomyosins (8), and further support for this comes from physiological studies that correlate the fiber type composition and contraction parameters for different muscles in mice (33). In addition, protein studies indicate that there is a IIx (24) or IId (40) and a superfast myosin HC (19). This highlights the need to use gene probes of proven specificity and the need to more fully characterize the skeletal myosin and other protein genes before drawing firm physiological conclusions from the molecular biology approach.

DISCUSSION

The mutability of mammalian skeletal fiber types was demonstrated by cross-innervation (5) and by chronic stimulation (45), and it was generally accepted that the motor impulse pattern, that is to say low-frequency repetitive stimulation, was responsible for slow muscle cell-phenotypic expression. However, more recently, it was

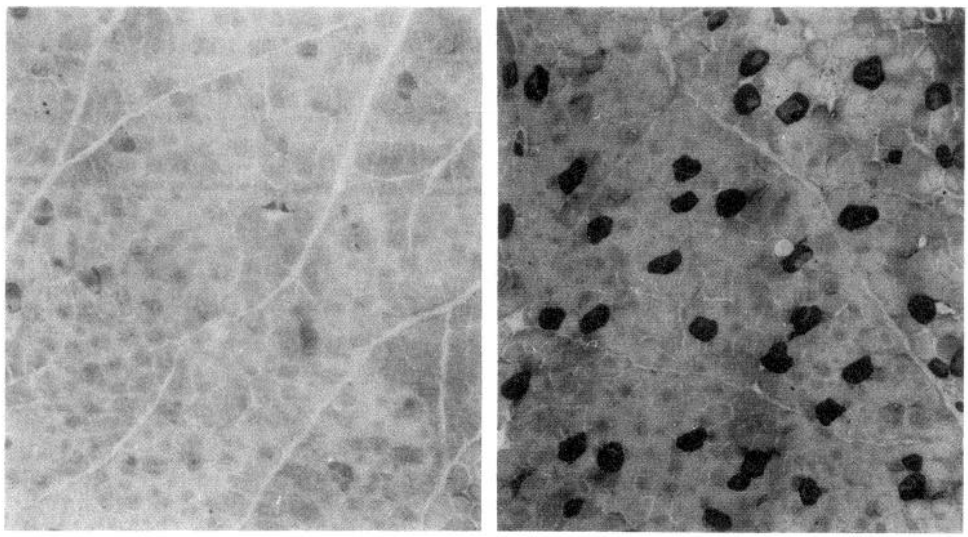

Fig. 3. Staining of transverse sections from the distal segment of the stretch-stimulated (30 Hz intermittent) rabbit tibialis anterior (TA) using a rabbit monoclonal antibody to slow skeletal myosin heavy chains. *Left*: control muscle; *right*: stretch/stimulated muscle. The antibody was donated by Dr. G. K. Dhoot of the Royal Veterinary College; it was visualized by the immunoperoxidase reaction. After 4 days of stretch and stimulation many more fibers in the rabbit tibialis anterior are seen to be expressing slow type myosin (dark staining).

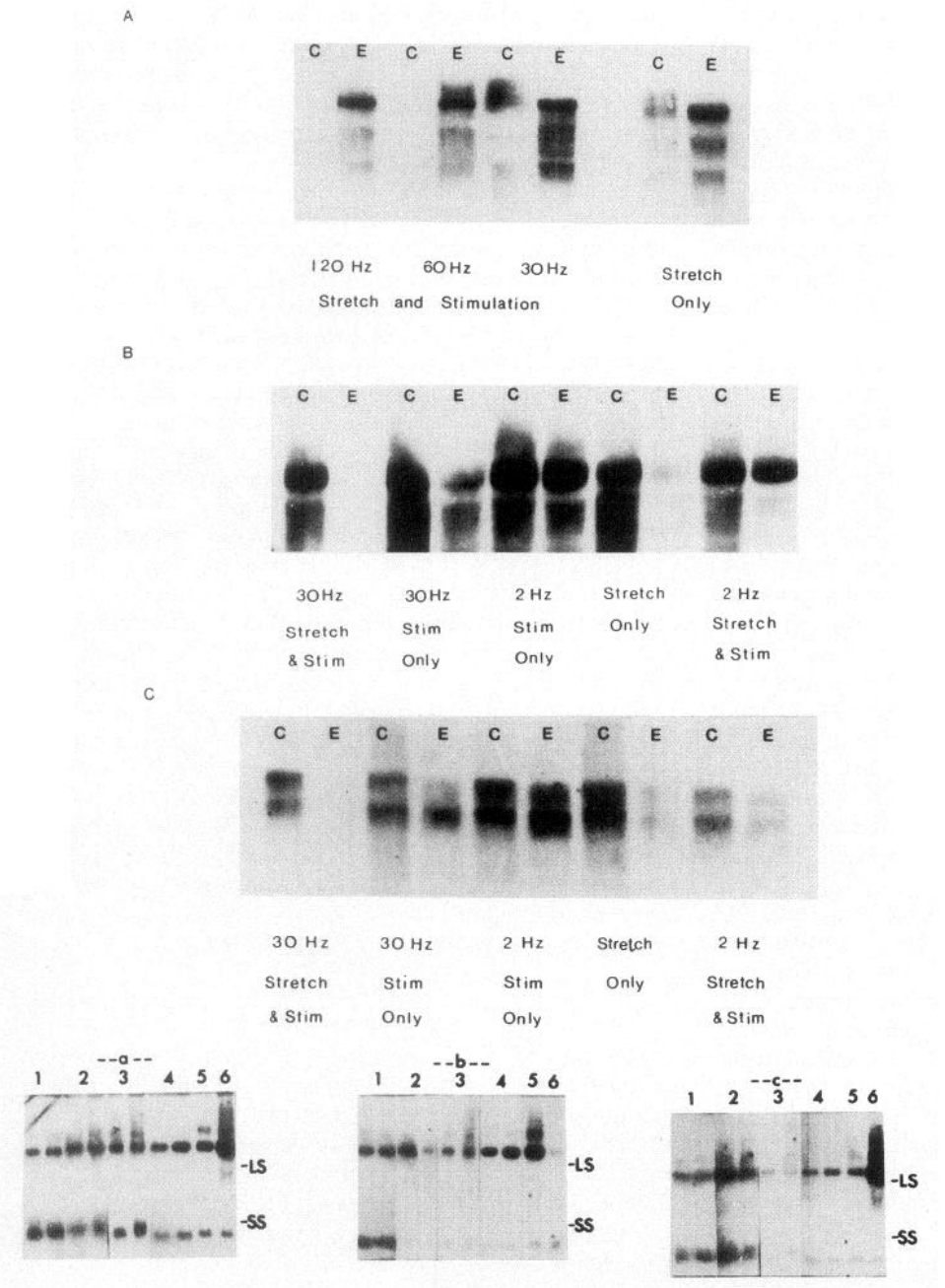

Fig. 4. *Top*: Northern blots using RNA from normal (C) and from stretched and stimulated rabbit TA muscles after 4 days (E). A: β-cardiac general slow probe showing activation of slow myosin HC. *B*: fast IIb myosin HC probe showing repression of this gene with stretch and/or stimulation. *C*: myosin light chain 1 and 3 probe showing repression of the myosin HC genes with stretch and/or stimulation. The 2 bands in the control muscles represent the message for the 2 light chains that are encoded by the same gene. Blots were carried out under moderate stringency conditions and were overexposed to determine the levels of repression of the fast IIb genes in the experimental muscles. *Bottom*: Northern blots using (*a*) pMHCβ 174-derived probe (specific for the coding region of β-myosin heavy chain, slow) and low-stringency washing conditions; (*b*) a pMHC 20–24-derived probe (specific for the coding region of the IIb myosin heavy chain fast) and medium-stringency washing conditions; and (*c*) a pMHCβ 174 *Sac*1-derived probe and medium-stringency washing conditions. All the blots for experimental and control muscles were carried out simultaneously and in duplicate, but the filters were cut and regrouped. *1*, TA stretched only; *2*, TA stretched and stimulated (30 Hz intermittent); *3*, TA stimulated only (30 Hz intermittent); *4*, TA control; *5*, extensor digitorum longus; *6*, soleus. The 2 lanes shown for each TA muscle represent independently processed samples. All blots were results of an 18-h exposure. The amount of RNA loaded per lane was chosen after initial blots of various dilutions such that they contained similar amounts of actin and myosin heavy chain message. LS, position of large ribosomal subunit (23 S); SS, position of small ribosomal subunit (16 S). All probes were labeled with ^{32}P. [From Goldspink et al. (14).]

shown that intermittent 60-Hz chronic stimulation for 5 wk was very effective in transforming fast muscles into slow muscles, as judged by the appearance of slow myosin light chains and histochemical staining (47).

The conclusions drawn from our data are in accord with these latter findings and indicate that it is those stimulation regimes that cause maximal force generation, particularly when they are combined with stretch, that switch on the slow genes and repress the fast genes. Repression of the fast IIb myosin HC has also been reported during chronic stimulation of the rabbit tibialis anterior (4).

Our work in this area differs from the use of cross-innervation or chronic stimulation techniques because in these cases the muscles are not increasing in size and do not induce the rapid rate of protein synthesis that results from stretch combined with stimulation. In the latter case the muscle is rapidly producing many new actin and myosin filaments in series and in parallel (12). A change in the type of protein isoform that is being expressed can be, therefore, readily detected in days rather than weeks (13). That is to say, the rates are not limited, as they presumably are in chronically stimulated muscle, to the protein turnover rates that normally occur in mature muscle tissue. Indeed, chronic stimulation experiments in which the muscle is not stretched result in considerably atrophy. As mentioned, fast myosin HC gene transcription can be elicited in normally slow muscle when the muscle is immobilized in the shortened position (29) and is undergoing atrophy. This suggests that muscle fiber growth is under separate control to muscle fiber-phenotype determination. Booth and coworkers (1, 7) found that levels of the actin mRNA remained very high during denervation atrophy, which

suggests that growth regulation is at the posttranscriptional levels. With the stretch-stimulation model, much new myosin is synthesized by the TA muscle in the mature animal within a very short period (11). This result is interesting, because apparently the synthetic machinery can still be activated by appropriate mechanical signals even in adult muscle, and questions arise as to what changes occur to permit such a rapid increase in synthetic rate. While investigating these questions (14), a significant increase in total RNA was observed (250%). The presence of many more ribosomes would presumably allow in much more of the available myosin message being translated into protein.

Protein studies have indicated that there is sequential myosin HC gene expression during muscle development (5, 6) and that the predominant protein isoforms change from embryonic, to neonatal, to adult fast. It seems that all muscle fibers stay phenotypically fast unless they are subjected to stretch and isometric force development. As shown by the soleus muscle that is immobilized in the shortened position (29) or subjected to hypogravity (39) or denervated by spinalization (24), the muscle reverts to expressing the fast myosin genes unless it is receiving these mechanical stimuli, under which circumstances the fast myosin genes are repressed. When the muscle is not subjected to stretch or force generation the fast myosin genes are expressed by default, which is essentially the same conclusion as drawn by Swynghedauw (48). He reviewed the conditions under which the fast myosin HC gene is expressed, and refers to the fast type gene as the endogenous gene, but we prefer the term "default gene." Certainly, the main regulation in the expression of the slow phenotype seems to depend on the repression of the fast type as much as the activation of the slow type genes. Other subsets of genes may be controlled in a similar fashion, including mitochondrial and cytoplasmic enzyme genes. For example, Williams et al. (61) reported that 10 Hz of chronic stimulation of the rabbit TA muscle for 21 days resulted in a fivefold increase in cytochrome *b* mRNA but a fourfold reduction in the levels of aldolase mRNA.

The details of the molecular mechanism(s) involved in isoform gene switching are not known. Two possible mechanisms spring to mind, including transient changes in internal calcium levels and metabolic signals such as the depletion of ATP and the change in the phosphorylation potential of the muscle cell. If calcium is involved in cellular signaling related to muscle gene activation, the system may be frequency rather than amplitude dependent (3). With respect to metabolic signals, Moerland et al. (35) have shown that administration of a creatine analogue induces significant changes in the type of myosin protein isoforms expressed in the mouse soleus and in the extensor digitorum longus muscles (from fast myosin light chains 1 to 3). The magnitude and rapidity of the switch in isoform gene transcription and the synthesis of much new protein make the stretch-stimulation model a good system for investigating the mechanical signals, second messengers, and transcription factors involved in muscle fiber-phenotype determination as well as in the control of muscle cell growth. The regulatory or early genes that code for putative transcription factors proteins such as c-*fos* and c-*myc* are also being studied (22, 57), but we still need to know what regulates the regulators.

As an adaptive mechanism, the switch in expression from the fast to the slow type genes under conditions of static overload or repetitive contraction makes physiological sense. It can be looked on as an adaptation for increased economy, since the slow myosin HC has a lower specific ATPase activity and uses less energy in maintaining isometric force and in slow repetitive movements (10, 11). Further work is necessary using more refined techniques in physiology and molecular biology to define the nature of the link between the mechanical signals and the activation and repression of subsets of muscle genes.

This work was funded by a grant from the National Aeronautics and Space Administration, the Wellcome Trust, the Agriculture and Food Research Council, and the Muscular Dystrophy Group of Great Britain. G. Goldspink received a travel grant from Pfizer to attend one of the conferences. G. F. Gerlach was supported by a grant from Natural Environmental Research Council.

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