Adaptation of Skeletal Muscle to Increased Contractile Activity

EXPRESSION OF NUCLEAR GENES ENCODING MITOCHONDRIAL PROTEINS*

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An increase in mitochondrial biogenesis in mammalian cells requires a coordinated increase in the expression of a number of nuclear genes that encode mitochondrial proteins. To examine the regulatory mechanisms involved, we used specific anti-sense RNA probes to estimate the cellular concentrations of mRNA transcripts of two such nuclear genes in rabbit tibialis anterior muscles subjected in vivo to 10–21 days of indirect electrical stimulation. The unstimulated contralateral muscle in the same animals provided a base line for comparison. Change in expression of mitochondrial proteins was assessed in terms of the enzymatic capacity of mitochondrial enzymes involved in the tricarboxylic acid cycle or in the electron transport chain. Since the maximum catalytic capacities of mitochondrial enzymes involved in the tricarboxylic acid cycle or in the electron transport chain increase in concert under such conditions (1, 3, 4), there must be mechanisms within the muscle cell that coordinate the expression of the many genes involved. The purpose of this study was to determine the stages of protein synthesis at which regulatory factors modulate the expression of nuclear genes encoding mitochondrial proteins. To this end, we have made use of specific probes to estimate the cellular concentrations of mRNA encoding subunits of the mitochondrial enzymes involved in the tricarboxylic acid cycle or in the electron transport chain.

MATERIALS AND METHODS

Electrical Stimulation and Preparation of Tissues—Miniature pulse generators were implanted in 11 adult New Zealand White rabbits, with the electrodes placed adjacent to the common peroneal nerve of one hind limb. Details of the operative procedure were as published previously (4). Activation of the pulse generator produced a continuous train of stimuli at 10 Hz, resulting in pulsatory contractions of the anterior compartment muscles of the operated limb. This procedure had no effect on body weight, or on eating, sleeping, or ambulatory behavior. Electrical stimulation was continued for 9–10 days (n = 4) or 21 days (n = 6). An additional animal, in which the pulse generator was inserted but not activated, served as a sham-operated control. As described previously (4), tissues were obtained by dissection under anesthesia, rinsed free of blood in sterile saline, frozen in liquid nitrogen, and stored at -70 °C until used for biochemical analysis.

Enzymatic Analyses, RNA and DNA Extractions, Hybridization Probes, and Hybridization Methods—As a marker for changes in mitochondrial biogenesis, the maximum catalytic capacities of the mitochondrial matrix enzyme citrate synthase and of the inner membrane enzyme cytochrome oxidase were measured in detergent-solubilized homogenates of muscle. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

If a skeletal muscle is subjected to an exercise pattern consisting of repeated contractions for 30 min or more per day, increases are observed in the fractional volume of mitochondria, enzymatic capacity for oxidative metabolism, and resistance to fatigue (1). Chronic electrical stimulation of a muscle via its motor nerve represents a more extreme functional demand which elicits changes on an even greater scale (2). This adaptive response includes both quantitative and qualitative changes in the expression of genes encoding proteins involved directly in muscle contraction and in the generation of energy for contraction (1–3). Since the maximum catalytic capacities of mitochondrial enzymes involved in the electron transport chain or in the tricarboxylic acid cycle increase in concert under such conditions (1, 3), there must be mechanisms within the muscle cell that coordinate the expression of the many genes involved. The purpose of this study was to determine the stages of protein synthesis at which regulatory factors modulate the expression of nuclear genes encoding mitochondrial proteins. To this end, we have made use of specific probes to estimate the cellular concentrations of mRNA encoding subunits of the mitochondrial enzymes involved in the tricarboxylic acid cycle or in the electron transport chain. Since the maximum catalytic capacities of mitochondrial enzymes involved in the tricarboxylic acid cycle or in the electron transport chain increase in concert under such conditions (1, 3, 4), there must be mechanisms within the muscle cell that coordinate the expression of the many genes involved. The purpose of this study was to determine the stages of protein synthesis at which regulatory factors modulate the expression of nuclear genes encoding mitochondrial proteins. To this end, we have made use of specific probes to estimate the cellular concentrations of mRNA encoding subunits of the mitochondrial enzymes involved in the tricarboxylic acid cycle or in the electron transport chain.

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previously (4). RNA concentrations were calculated from the A_{260} of the solubilized extracts, assuming 40 μg/ml to be equivalent to 1.0 absorption unit. DNA concentrations were determined by the diaminobenzic acid-fluorescence reaction (8).

In preparation for hybridization studies, nucleic acids were immobilized on nitrocellulose filters following either direct blotting in a vacuum filtration manifold (dot blots) or transfer after electrophoresis in agarose gels (Northern and Southern blots).

Hybridization probes were prepared by two methods. In the one, plasmid DNA was labeled with [32P]thymidine triphosphate to a specific activity of greater than 1 × 10^8 dpm/μg by nick translation. In the other, anti-sense RNA labeled to a specific activity of greater than 1 × 10^8 dpm/μg was produced (9) by incorporating [32P]uridine triphosphate into RNA transcripts produced in vitro with SP6 polynuclease (Promega Biotec) and the orientation of the insert in transformant clones was determined by restriction mapping with PstI to determine the sense versus anti-sense of the insert relative to the SP6 promoter. A clone containing the insert in the anti-sense orientation (SP6-14/2) was then utilized as the template for in vitro transcription to produce labeled probes for hybridization studies.

Plasmid pBR142 containing CDNA encoding subunit I of the F°-ATPase (10) cloned from Saccharomyces cerevisiae was a gift from Dr. Michael Douglas (University of Texas Health Science Center, San Antonio). A BamHI restriction fragment of pBR142 containing 2390 base pairs of β−ATPase genomic DNA was inserted into the BamHI site in the polylinker segment located 3′ to the SP6 promoter of pSP65 (Promega Biotec). After transformation of Escherichia coli, plasmid DNA extracted from clones produced from single colonies was analyzed by restriction mapping with PvuII to determine the orientation of the insert in transforming clones was determined by restriction mapping with EcoRI. A clone bearing the insert in the anti-sense orientation (SP6-14/2) was then utilized as a template for generating labeled anti-sense RNA probes by transcription in vitro with SP6 polynuclease.

Aldolase A mRNA was detected in Northern blots and RNA dot blots by hybridization to a 735-base pair probe fragment of pBR223, provided by Dr. Dean Tolan (University of California, Berkeley), that contains a portion of the protein coding region of aldolase A cDNA from rabbit muscle (12).

Cytochrome b mRNA and mitochondrial DNA were detected by hybridization to a 9.2-kb BamHI fragment of pMM26, a segment of mitochondrial DNA prepared originally by Dr. Ian Craig (University of Oxford) from mouse liver and containing the complete coding region of cytochrome b (13).

The identities of the plasmids used to produce probes in this study were confirmed by restriction mapping (10–13).

DNA-DNA and RNA-DNA hybridizations were performed at 42 °C for 24 h in hybridization buffer containing 50% formamide, 0.3 M sodium chloride, 30 mM sodium citrate, pH 7.0, 50 mM sodium phosphate, 0.1% sodium dodecyl sulfate, 0.2% bovine serum albumin (Fraction V), 0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 400 μg/ml salmon sperm DNA. RNA-RNA hybridizations were performed at 55 °C in 50% formamide containing 50 mM sodium phosphate, pH 6.5, 0.8 M sodium chloride, 0.5 mM EDTA, 0.1% sodium dodecyl sulfate, 0.05% polyvinylpyrrolidone, 0.05% bovine serum albumin (Fraction V), and 400 μg/ml salmon sperm DNA. Filters from DNA-DNA or DNA-RNA hybridizations were washed for 2 h at 42 °C in 50 mM sodium phosphate, 30 mM sodium citrate, pH 7.0, 0.1% sodium dodecyl sulfate with three changes of buffer. Filters from RNA-RNA hybridizations were washed for 1 h at 60 °C with three changes of buffer containing 20 mM sodium phosphate, pH 6.5, 50 mM sodium chloride, 1 mM EDTA, and 0.1% sodium dodecyl sulfate.

Filters were exposed to Kodak XAR-5 film with one intensifying screen and stored at −70 °C. To estimate the relative concentrations of mRNA encoding β−ATPase, cytochrome oxidase VIC, aldolase A, or cytochrome b, the intensities of the hybridization signals from dot blots loaded with serial dilutions of RNA extracted from stimulated and control muscles were compared by numerical integration of individual dots. In pilot studies, such estimates conducted on RNA extracted in parallel from duplicate samples of the same muscle varied by 6%. In addition, recovery of a

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**TABLE I**

| Duration of stimulation (days) | 9–10 (n = 4) | 21 (n = 6) |
|-------------------------------|-------------|-----------|
| Muscle, wet weight            | 82 ± 6      | 75 ± 6    |
| Protein/g, wet                | 102 ± 12    | NS\*       | 102 ± 12   | NS       |
| DNA/g, wet                    | 87 ± 5      | NS        | 117 ± 19   | NS       |
| RNA/g, wet                    | 142 ± 41    | NS        | 183 ± 28   | p < 0.05 |
| Aldolase A, V_{max}           | 66 ± 6      | p < 0.01  | 26 ± 3     | p < 0.001 |
| Citrate synthase, V_{max}     | 213 ± 28    | p < 0.05  | 547 ± 66   | p < 0.001 |
| Cytochrome oxidase, V_{max}   | 215 ± 12    | p < 0.001 | 412 ± 50   | p < 0.001 |

\* NS, not significant.

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The abbreviation used is: kb, kilobase pair.
NORTHERN BLOTS PROBED WITH SP6-14β2

| Day  | C   | S   |
|------|-----|-----|
| 10   | 10  | 10  |
| 20   | 10  | 20  |

FIG. 1. Northern blots of muscle RNA probed with anti-sense RNA transcribed *in vitro* from SP6-14β2, which contains genomic DNA encoding subunit β of F₁-ATPase. The total amount of RNA added to each lane (10 or 20 µg) is indicated. A stronger hybridization signal was detected in extracts of muscles stimulated electrically (S) for 9 or 21 days compared with control muscle (C). These autoradiographs were exposed either for 48 h (left) or 16 h (right).

NORTHERN BLOTS PROBED WITH SP6-pCO89

| Day  | C   | S   | A⁺ |
|------|-----|-----|----|
| 10   | 10  | 10  | 1  |
| 20   | 10  | 20  | 2  |

FIG. 2. Northern blot of muscle RNA probed with anti-sense RNA transcribed *in vitro* from SP6-089, which contains cDNA encoding subunit VIC of cytochrome oxidase. The total amount of RNA loaded in each lane is indicated (microgram). Hybridization signals from all bands were increased in RNA extracted from stimulated (S) versus control (C) muscles. This blot was derived from animals stimulated for 21 days. The autoradiograph was exposed for 14 h. The lowest molecular weight form appears to represent the mature mRNA, since only this form was evident in polyadenylated RNA (poly(A⁺)).

FIG. 3. RNA dot blot probed with anti-sense RNA transcribed *in vitro* from SP6-14β2. RNA extracted from stimulated (S) or control (C) muscles was applied directly in the indicated amounts to nitrocellulose filters and hybridized as described in the text. Each vertical set of three concentrations of RNA represents an extract from a single muscle, and S and C muscles from the same animal are shown side by side. The duration of electrical stimulation of each S muscle received is indicated either above each column (upper three rows) or below each column (lower three rows). This autoradiograph was exposed for 6 h.

that this contains the mature message (Fig. 2). Stimulation brought about an increase in hybridization that was similar in all bands.

As published previously from this laboratory (4), nick-translated aldolase A cDNA hybridized to a single 1.4-kb band in Northern blots of both total and polyadenylated rabbit muscle RNA and the nick-translated mitochondrial DNA probe identified a 1.2-kb band compatible with cytochrome b mRNA.

Scintillation counting of RNA dot blots (Fig. 3) probed with each of the probes yielded a quantitative comparison of the relative amount of βF₁-ATPase, cytochrome oxidase subunit VIC, aldolase A, and cytochrome b mRNA in stimulated and control muscles, the results of which are summarized in Table II.

Expressed as a proportion of total RNA per unit of tissue mass, wet weight, or per fiber (assuming no change in the number of fibers per muscle), mRNA transcribed from the nuclear genes encoding βF₁-ATPase and cytochrome oxidase subunit VIC was significantly increased by electrical stimulation. Messenger RNA transcribed from the mitochondrial gene encoding cytochrome b was also increased and the magnitude of the increase was greater than that of βF₁-ATPase and cytochrome oxidase subunit VIC mRNA. In contrast,
mRNA encoding aldolase A (a nuclear gene product) fell in stimulated muscle to a level only one-fifth of that present in control tissue.

When the mRNA data were expressed per μg of total DNA, changes in cytochrome b and aldolase A mRNA remained statistically significant, but the trends toward greater expression of βF₂-ATPase and cytochrome oxidase mRNA (approximately 1.5-fold increases) in stimulated muscle were no longer significant at the p < 0.05 level.

In dot blots prepared with equivalent amounts of polyadenylated RNA, the hybridization signal observed when PF₁-ATPase antisense RNA was used as a probe was 260 ± 28% of the control value in muscles stimulated for 21 days. The corresponding result when cytochrome oxidase subunit VIC genes were probed was 260 ± 28% of the control value. These values are similar to the results obtained by analysis of total RNA (Table II), we conclude that stimulation produced no major changes in the ratio of polyadenylated to total RNA within the muscle fibers.

**DISCUSSION**

Long term electrical stimulation of mammalian skeletal muscle is a powerful stimulus for mitochondrial biogenesis, producing changes similar to, but more extreme than, those induced by endurance exercise training (1-4, 14, 15). The regulatory mechanisms that bring about the accompanying changes in gene expression could operate at a number of different stages of protein synthesis to modulate one or more of the following: efficiency of transcription, the rate of RNA processing, the stability of the mRNA, the efficiency of translation, the rate of post-translational modification or transport, and the stability of the protein product.

The present study enables us to draw conclusions about the relative importance of these mechanisms for the regulation of two nuclear genes encoding mitochondrial proteins. First, elevated levels of mRNA transcribed from these genes are present in skeletal muscles subjected to a powerful stimulus to augment mitochondrial biogenesis. At this time we are unable to distinguish between accelerated rates of transcription, alterations in RNA processing events, or altered stability of the mRNA as the basis for the elevated mRNA levels. Likewise, we cannot yet exclude a contribution of increased multinuclearity or rearrangements of gene structure within pre-existing nuclei to this response. However, the specific pretranslational regulatory events that occur in response to electrical stimulation and that contribute to enhanced expression of these nuclear genes encoding mitochondrial proteins must involve selective processes, since other nuclear genes (e.g. aldolase A) are affected quite differently.

Second, changes in βF₂-ATPase and cytochrome oxidase subunit VIC mRNA are not as large as those in mitochondrial volume fraction, Vₘ₉₉, of mitochondrial marker enzymes, and mRNA transcribed from mitochondrial genes (e.g. cytochrome b). This finding suggests that pretranslational regulation alone is insufficient to account fully for the changes in expression of the protein products of the βF₂-ATPase and cytochrome oxidase subunit VIC genes. Enhanced translational efficiency, accelerated transport of these proteins from their cytoplasmic sites of synthesis across the mitochondrial membranes, or increased stability of the proteins may also be required to support the accelerated mitochondrial biogenesis induced by electrical stimulation.

The disparity between the magnitude of the increase in βF₂-ATPase and cytochrome oxidase subunit VIC (2-3-fold) and our estimates of the magnitude of increase of the protein products of these genes (4-6-fold) in response to electrical stimulation contrasts with our earlier findings relating not only to cytochrome b, encoded by a mitochondrial gene, but to aldolase A, a nuclear gene product. Both cytochrome b mRNA (increased 5-6-fold) and aldolase A mRNA (decreased 4-5-fold) changed in direct proportion to our estimates of the change in the protein products of these genes. Therefore, in the case of cytochrome b and aldolase A, pretranslational regulation seems sufficient to account fully for the changes in expression at the protein level, a conclusion that contrasts with our current results concerning βF₂-ATPase and cytochrome oxidase subunit VIC.

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