THE REGULATION OF GENE EXPRESSION IN STRIATED MUSCLE DURING CONDITIONS OF ALTERED CONTRACTILE ACTIVITY

Michael K. Connor

A thesis submitted to the Faculty of Graduate Studies of York University in partial fulfilment of the requirements for the degree of

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ABSTRACT

Contractile activity represents a potent stimulus for the induction of alterations in skeletal muscle phenotype. The dynamic nature of gene expression requires tight regulatory mechanisms that match skeletal muscle protein profile to metabolic demand. Mitochondrial biogenesis occurs in muscle in response to altered contractile activity and is central to many of the adaptations that occur during this period. The mitochondrion is comprised of proteins that are encoded in the nuclear genome as well as those which are encoded within the mitochondrial DNA. Thus, the process of mitochondrial biogenesis is a complex one that requires communication between the nucleus and mitochondrion in order to properly coordinate organelle synthesis. There are numerous studies which have investigated activity-induced mitochondrial biogenesis however, the stable nature of the mRNAs encoding mitochondrial proteins has made the elucidation of the mechanisms underlying organelle biogenesis difficult. Consequently, there exists a need for more sensitive techniques in order to fully understand the response of the genes encoding mitochondrial proteins to contractile activity. In addition, there needs to be a better appreciation of the relationship between how muscle responds to both increased and decreased contractile activity, as it does not appear that one response is simply the opposite to the other.

Thus, my thesis was designed to fulfill the following purposes. 1) To clarify some of the ambiguities that exist in the response of skeletal muscle to short-term microgravity exposure and determine at which level of gene expression that adaptations are regulated. Furthermore, it is known that the cardiovascular system is subjected to many stressors during

microgravity exposure and I wish to determine the molecular response of cardiac muscle to spaceflight. 2) The stable nature of skeletal muscle gene products which encode mitochondrial proteins has prevented the elucidation of the mechanisms responsible for the changes in gene expression following increases in contractile activity. My experiments were directed towards developing more reliable methods for examining the expression of nuclear genes encoding mitochondrial genes. 3) Current *in vivo* models for studying the effects of contractile activity on gene expression do not allow for the precise manipulation of the intracellular environment that is necessary to determine the putative signals that initiate alterations in gene expression. I wished to develop a contracting cell culture model to better investigate the mechanisms which underlie the response of skeletal muscle to increased contractile activity.

In this regard, my studies utilized both whole animal and cell culture models. Steady-state mRNA and protein levels were measured, in order to determine the extent of the activity-induced mitochondrial adaptations in addition to providing some insight into the level of regulation of this process. By measuring the transcription and degradation of specific mRNAs, a better understanding of how increased contractile activity elicits adaptations in the expression of genes encoding mitochondrial proteins was developed. In addition, a cell culture model of increased muscle contraction allowed for control of specific proteins and a better definition of the transcriptional regulation of nuclear-encoded mitochondrial genes. Also, the inhibition of muscle contraction at various levels allowed for the determination of the signal(s) responsible for activity-induced skeletal muscle

mitochondrial biogenesis.

Following exposure to microgravity, tissue-specific adaptations in the expression of mitochondrial proteins in skeletal and cardiac muscle were evident. In contrast, the response of skeletal muscle to increased contractile activity was explored to provide a clear definition of the molecular mechanisms that initiate mitochondrial biogenesis. Using direct muscle gene injection and an *in vitro* mRNA decay system , sequential activity-induced elevations in both mRNA transcription and stability, respectively, were evident. Also, cytochrome c expression was dependent on the type of stimulation (i.e. continuous vs. intermittent), suggesting a role of the recovery phase in this adaptation. Finally, using a cell culture model it was determined that activity-induced elevations in the transcription of the nuclear-encoded cytochrome c gene could be mapped to a specific region. This adaptation was mediated by specific proteins binding within this region and was dependent on cross-bridge cycling.

Thus, mitochondrial adaptations in muscle during conditions of increased and decreased contractile activity display a unique, tissue-specific response. In addition, the data presented herein provide the groundwork for further investigation into the molecular basis of activity-induced skeletal muscle mitochondrial biogenesis.

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LIST OF ABBREVIATIONS

ARE	AU-rich element
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BAPTA-AM	1,2-bis(*o*-Aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester
BDM	2,3-butanedione monoxime
β-gal	Beta-galactosidase
bp	Base pair
CAT	Chloramphenicol acetyltransferase
ctl	Control
CYTOX	Cytochrome c oxidase
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSIF	DRB-sensitivity inducing factor
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic acid
ELAV	Embryonic lethal abnormal vision
GAPDH	Glyceraldehyde-3-phosphate

	dehydrogenase
GM-CSF	Granulocyte-macrophage colony stimulating factor
HSP	Heat shock protein
Inr	Initiator
IRE	Iron response element
IRP	Iron-regulatory protein
kDa	Kilodalton
MDH	Malate deyhdrogenase
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
mtHSP	Mitochondrial heat shock protein
mtTFA	Mitochondrial transcription factor A
nTEF	Negative transcription elongation factor
PABP	Poly (A) binding protein
PIC	Pre-initiation complex
POLII	RNA polymerase II
pTEF	Positive transcription elongation factor
RRM	RNA recognition motif
RSV	Rous sarcoma virus

stim	Stimulated
TA	Tibialis anterior
TAF	TBP-associated factor
TBP	TATA binding protein
TTX	Tetrodotoxin
UTR	Untranslated region

REVIEW OF RELATED LITERATURE

1. GENE EXPRESSION

Differences in the levels of proteins among mammalian tissues result from altered expression of these genes within these tissues (Connor et al. 1996, Hood, 1990). There are numerous factors which contribute directly to tissue-specific gene expression, and these factors are also likely responsible for adaptations occurring within a tissue in response to an external stressor. Although there are many potential levels of regulation for the expression of nuclear and mitochondrial genes, I will limit this review to gene transcription and mRNA stability.

1.1 Transcription of Nuclear Protein-coding Genes

The majority of the genetic information contained in a eukaryotic cell is located within the nuclear genome, with a small number of proteins being encoded by the mitochondrial DNA. Within the nucleus, the multi-subunit enzyme RNA polymerase II (POLII) is responsible for the transcription of protein-coding genes into messenger RNA (mRNA; Myer and Young, 1998). The POLII holoenzyme, which is comprised of subunits responsible for DNA-binding in addition to those involved in the regulation of transcriptional activity, comprises the heart of the core transcriptional complex and is the target for many transcription co-factors (Myer and Young, 1998, see below). When in a non-dividing state, nuclear DNA is complexed with an octamer of core histone proteins (H2A, H2B, H3 and H4; Wolffe, 1997) which bind DNA in their N-termini (Hansen et al., 1998). This complex, termed the nucleosome, prevents POLII access to the DNA, thus preventing gene transcription. In order to transcribe nuclear-encoded genes, the nucleosome must be

disrupted, thereby exposing the promoter regions to the transcriptional machinery (Edmonson and Roth, 1996). This is an ATP-dependent process that is facilitated by a family of proteins termed helicases (Eisen and Lucchesi, 1998). In addition, histone acetyltransferases will acetylate internal lysine residues of the core histone N-terminal domains, resulting in changes in histone conformation which disrupt the nucleosome and allow POLII and other transcription factors access to the gene promoters (Kuo and Allis, 1998). This acetylation is often transient and can be reversed by the action of histone deacetylases (Kuo and Allis, 1998). Thus, the nature of the nucleosome allows for the selective regulation of the expression of nuclear-encoded genes.

1.1.1 Core Transcriptional Complex

Many protein-coding genes contain a T-A rich region within their promoters termed a TATA box. This TATA box is commonly found approximately 30 basepairs (bp) upstream of the transcription start site, and has been shown to be the site within the promoter at which the transcriptional apparatus binds (Ptashne and Gann, 1997; Zawel and Reinberg, 1995). Although POL II alone can bind to the TATA box, this occurs at a very low and somewhat inefficient rate (Smale, 1997). However, there is a large family of transcription factors (TFIIs) which are necessary to increase the efficiency of both the DNA binding and transcriptional elongation of POLII (Bell and Tora, 1999; Hahn, 1998; Myer and Young, 1998; Tansey and Herr, 1997; Zawel and Reinberg, 1995). The TATA-binding protein (TBP) is a 38-kDA protein which, as its name suggests, binds to the TATA box and is very strongly associated with a large group of TBP-activating factors (TAFs) collectively termed

TFIID (Hoffmann et al., 1997; Lewin 1990; Tansey and Herr, 1997). The TBP-TAF complex is very stable, with dissociation occurring only under denaturing conditions (Tanese et al., 1991). Interestingly, TFIID is also thought to play a major role in the initiation of transcription of TATA-less promoters (discussed in more detail below). The nature of TBP binding to the TATA box is altered by direct TAF protein-protein interactions. TFIID binding to the TATA box is stabilized by interaction with amino acids 217-240 of the protein TFIIA (Kang et al., 1995). Upon binding to the TATA box TFIID interacts with TFIIB, a protein that spans approximately 30 bp and acts to direct the growing transcription pre-initiation complex (PIC) to transcription start site (Ha et al., 1993). TFIIB interacts with TFIIF, the factor responsible for the recruitment of POLII into the PIC. Thus, TFIIB can be thought of as a molecular bridge between TFIID and POLII (Flores et al., 1991). There are 2 other proteins, TFIIE and TFIIH which associate with the PIC however, they are more essential for promoter clearance and the nucleotide excision repair (Aboussekhra et al., 1995; Dahmus, 1996; Zawel and Reinberg, 1995).

1.1.2 TATA-less Promoters

There are some eukaryotic protein-coding genes, such as cytochrome c (Scarpulla et al., 1981), which do not contain a TATA box within their promoter regions yet are efficiently transcribed within the nucleus (Smale, 1997). Thus, the TATA box is not the only region which can direct the transcription of mRNA. These TATA-less promoters contain an initiator (Inr) sequence which encompasses the sequences between -6 to +11 bp and contains a loosely defined consensus sequence (CANT; Smale, 1997). This consensus sequence,

which overlaps the transcription start site (A being located at +1 bp), must be surrounded by at least a few pyrimidines to confer accurate transcriptional activation of these genes (Jahavery et al., 1994; Lo and Smale, 1996). Interestingly, Inr regions are located in many TATA-containing promoters and appear to increase transcriptional efficiency (Means and Farnham, 1990). When an Inr region was placed downstream of either a TATA box or multiple Sp1 binding sites, transcriptional activity was greater than that observed in the absence of the Inr (Means and Farnham, 1990). Thus, the Inr is similar to the TATA box in that 1) both of these regions can direct accurate transcription of protein-coding genes and 2) each can support high levels of transcription when subjected to an upstream activator (Smale, 1997). However, it is likely that there are still some unidentified components involved in Inr driven transcription and the full nature of the expression of these TATA-less promoters remains unknown.

1.1.2.1 TFIID

Similar to transcription from TATA-containing promoters, many TATA-less promoters drive transcription via the recruitment of TFIID to the transcription start site (Carcamo et al., 1991; Kaufmann and Smale, 1994; Means and Farnham, 1990; Verrijzer et al., 1995). However, it still remains unclear which component of the TFIID complex is responsible for Inr binding (Smale, 1997). It appears that the essential components of TFIID are TBP and two TAFs within the TFIID complex, TAF150 and TAF250, since a TBP-TAF150-TAF250 complex can initiate transcription from the Inr region *in vitro* (Verrijzer et al., 1995). It has been hypothesised that TFIID-Inr interactions are weaker than TFIID-

TATA interactions, since purified TFIID alone cannot bind Inr promoter sequences in the absence of an intact TATA box, as indicated by gel mobility shift analysis (Kaufmann and Smale, 1994) This suggests the involvement of other factors which stabilise TFIID-Inr interactions *in vivo*. A likely candidate involved in the stabilisation of TFIID binding to the Inr is TFII-I, one of the first proteins found to exhibit Inr binding (Roy et al., 1991).

1.1.2.2 TFII-I

TFII-I has been shown to replace the requirement for TFIIA in the PIC (see section 1.1.1. above). TFII-I is capable of binding to DNA containing a TATA box and an Inr and this persists even in the presence of a mutated TATA box suggesting the involvement of TFII-I in the transcription of TATA-less promoters (Roy et al., 1993). TFII-I can also stimulate transcription in the presence of TBP alone (Roy et al., 1991), although it remains likely that the entire TFIID complex is necessary for Inr driven transcription (Kaufmann and Smale, 1994; Verrijzer et al., 1995).

1.1.2.3 Other Factors

It has been speculated that POLII itself is capable of binding to the Inr (Carcamo et al., 1991). However, this hypothesis was based on experiments in which purified POLII, TBP, TFIIB and TFIIF were shown to bind Inr elements. Since the latter three components are not capable of binding to Inr regions, it was proposed that POLII was the protein responsible for Inr binding. Although this is a logical conclusion, no direct evidence has demonstrated that POLII alone can bind to the Inr. Another recently reported protein Yin Yang 1 (YY1) has been shown to possess a high affinity for sequences containing a CCAT

core, and will thus bind to a small subset of Inr containing promoters (Seto et al., 1991). Transcription of adenoassociated virus P5 promoter, which contains an Inr sequence but no TATA box, can be reconstituted *in vitro* by the addition of only YY1, TFIIB and POLII (Usheva and Shenk, 1994). There was no requirement for TBP or TFIID, and these proteins actually inhibited the YY1-dependent transcription initiation.

1.2. Transcriptional Elongation

To date much of the research directed at the analysis of gene transcription has focussed on the pre-initiation and initiation stages of this process. However, DNA transcription is comprised of two essential stages, initiation and elongation (Aso et al., 1995a; Shilatifard, 1998a). In recent years there has been an increase in the amount of research investigating transcriptional elongation, which has begun to help improve the understanding of how this process is controlled.

1.2.1. pTEFs

The initiation of mRNA transcript elongation is controlled by the actions of transcription elongation factors (Marshall and Price, 1995; Wada et al., 1998a). Positive transcription factors (pTEFs) have been purified from *Drosophila* extracts and yielded three activities, pTEFa, factor 2 and pTEFb (Marshall and Price, 1995). Although all three factors aid in transcript elongation, it appears that pTEFb is the only factor that is strictly required in that it supports elongation in the absence of the other two factors (Marshall and Price, 1995). pTEFb is comprised of two subunits which have been characterized as a 124 kDa cyclin dependent kinase (CDK9) and a 43 kDA cyclin (cyclinT; Peng et al., 1998). It has

also been shown that pTEFb can phosphorylate the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (Dahmus, 1996; Marshall et al., 1996). pTEFb is thought to release the transcriptional complex from elongation arrest, possibly through phosphorylation of the CTD of the largest POLII subunit by the CDK9 subunit.

1.2.2. DSIF

A recently identified protein, termed DRB-sensitivity inducing factor (DSIF), is composed of 160 kDa and 14 kDa subunits. DSIF inhibits elongation in combination with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Wada et al., 1998a), a chemical which has previously been shown to inhibit mRNA synthesis. DSIF has been recently shown to interact with pTEFb. This is significant since pTEFb releases POLII from elongation arrest. Thus, the activities of these antagonistic proteins likely act to modulate the rates of transcriptional elongation (Wada et al., 1998b). In addition, DSIF has been shown to interact with a novel protein termed NELF (negative elongation factor), a protein comprised of five subunits which cooperates with DSIF to strongly inhibit POLII elongation (Yamaguchi et al., 1999). The smallest NELF subunit is identical to RD, a putative RNA-binding protein of unknown function. The relevance of this finding remains unknown and the relatively novel nature of the relationship between DSIF and NELF means that the understanding of their functions *in vivo* are speculative in nature. However, a better comprehension of this interaction is likely to evolve in the near future.

1.2.3. SII

SII is an ubiquitously expressed 38 kDa elongation factor that was discovered and

purified in the mid 1970s (Sekimizu et al., 1976). It has been established that POLII directed transcription is not a continuous process and that many transcription arrests occur during mRNA synthesis (Reines et al., 1996). During transcription POLII encounters many intrinsic arrest sites, which are typically composed of two or more closely spaced T-rich stretches in the template strand (Shilatifard, 1998a). SII acts to release POLII from its arrested state by allowing elongation to proceed through these arrest sites (Kerppola and Kane, 1991). Upon transcriptional arrest POLII undergoes a conformational change which leads to a loss of contact between the 3′ end of the growing transcript and the POLII catalytic site (Donahue et al., 1994). It is likely that SII promotes reiterative message cleavage and re-extension of the nascent transcript held within the POLII active site (Izban and Luse, 1992). Evidence suggests that although a physical interaction between SII and POLII is necessary for transcript cleavage, the enzymatic activity is contained within POLII itself (Izban and Luse, 1992; Reines, 1992).

1.2.4. The Elongin (SIII) Complex

The elongin/SIII complex of proteins is comprised of A, B and C subunits with molecular masses of 110, 18 and 15 kDa, respectively (Bradsher et al., 1993a; Bradsher et al., 1993b). Elongin A is the transcriptionallly active subunit while Elongins B and C are positive regulatory subunits, which form a stable complex capable of inducing the transcriptional activity of Elongin A (Aso et al., 1995b). Elongin C appears to be the true inducer of Elongin A activity, since these two proteins can interact in the absence of Elongin B. However, Elongin B is incapable of binding to Elongin A without the presence of

Elongin C, suggesting a chaperone-like function for Elongin B (Aso et al., 1995b). The elongin/SIII complex suppresses transient pausing of POLII, but the exact mechanism of action remains unknown. It has been shown that elongin B/C complex is tightly bound by the von Hippel-Landau (VHL) tumor suppressor protein (Duan et al., 1995) suggesting that these factors regulate gene expression by slowing the transcriptional activity of POLII. Patients with deficient levels of VHL protein exhibit an increased incidence of various types of cancer, likely a result of an increased POLII activity mediated by a disruption of the Elongin/SIII/VHL interactions.

1.2.5. ELL

ELL is an 80 kDa polypeptide, originally purified from rat liver nuclei, which contains no obvious structural motifs characteristic of transcription factors but is capable of inhibiting transcriptional pausing by POLII (Shilatifard et al., 1996). ELL contains an elongation activation domain as well as a novel domain which negatively regulates elongation *in vitro* (Shilatifard et al., 1997). If ELL is added to transcription reactions before the formation of the PIC, there is a reduction in the rate of transcription (Shilatifard et al., 1997). Patients with acute myeloid leukemia posses a deletion of the negative regulation domain of ELL which results in an increased promoter-specific transcription initiation. However, it has recently been discovered that ELL exists as a Holo-ELL complex comprised of other cellular proteins with an approximate mass of 210 kDa (Shilatifard, 1998b). The ELL-associated proteins (EAPs) which constitute this complex inhibit the negative effects of ELL, and the Holo-ELL complex acts to increase the rate of catalytic rate of transcription

elongation (Shilatifard, 1998b). Thus, it appears likely that one of the EAPs interacts with the negative transcription regulation domain of ELL and prevents transcription inhibition.

1.2.6. TFIIF

TFIIF exhibits a unique feature compared to the other general transcription factors in that it has the ability to affect POLII activity by altering both the rates of initiation and elongation (Reines et al., 1996). If POLII interacts with TFIIF prior to encountering a transcriptional arrest site, there appears to be a reduced likelihood that arrest will occur (Gu and Reines, 1995). It appears that although TFIIF action is unique from that of SII, there is a complementary relationship which exists between the two proteins. TFIIF reduces the chance that transcriptionally active complexes will pause and SII releases those which do experience transcription arrest.

1.3. Transcription Factors

It is well established that activity of the core transcriptional complex can be modulated by the activity of proteins which bind to the gene either in the promoter 5′ to the transcription start site or within the first intron (Calkhoven and Geert, 1996; Chambers et al., 1995; Darnell, 1982). The list of such transcription factors is extensive and the information known about many of these proteins is sufficient to produce a lengthy review for each one. As such I will limit my discussion to a relatively cursory explanation of c-Jun and Sp1, two transcription factors which have been implicated in the regulation of the cytochrome c gene in striated muscle (Evans and Scarpulla, 1989; Xia et al., 1998) and are thus relevant to this thesis.

1.3.1. c-Jun

The Jun protein is an inducible transcription factor which has been shown to respond to numerous cellular stimuli. A viral counterpart of this protein (v-jun) is encoded by the avian sarcoma virus 17 and the abbreviation of the Japanese term *ju-nama*, meaning 17, is the origin of the current nomenclature of Jun (Rahmsdorf, 1996). c-Jun is a basic leucine zipper protein that was originally identified as a 39 kDa moiety that immunoprecipitated with c-Fos (Curran et al., 1984, Curran et al., 1985). c-Jun binds to DNA as a dimer, either with another c-jun molecule (homodimer) or as a heterodimer with c-fos or other members of the Jun and Fos families. This c-jun dimer is capable of initiating transcription and is referred to as AP-1 (Bohmann et al., 1987). The consensus AP-1 binding sequence (TGAGTCA) is present in numerous cellular genes making c-Jun a potent regulator of gene transcription. To date, much of the evidence that has defined the regulation of c-Jun has shown that the activity of the protein is regulated mainly by post-translational modifications (Papavassilliou et al., 1995). For example, in non-treated cells c-Jun is phosphorylated on serine (SER) and threonine (THR) residues within the carboxy-terminus. Hyper-phosphorylation of these residues decreases the DNA-binding capacity of c-Jun, and mutations of these residues results in an enhancement of c-Jun transcriptional activation (Boyle et al., 1991; Hagmeyer et al., 1993). It has been shown that following treatment with a phorbol ester (Nikolokaki et al., 1993), protein kinase C can inhibit the activity of glycogen synthase kinase-3β (GSK-3β), which phosphorylates c-Jun in its C-terminus (Nikolokaki et al., 1993; Sutherland et al., 1993; Troussard et al., 1999). Thus, phorbol ester activation of c-Jun predominantly

involves removal of the basal inhibitory hyperphosphorylation and thereby activating the protein (Goode et al., 1992). Accordingly, under normal conditions c-Jun is heavily phosphorylated within its DNA-binding domain by GSK-3β, and the removal of this suppressive effect is one of the important events in c-Jun activation.

When in an activated state, in addition to being hypophosphorylated in its carboxy terminus, c-Jun is phoshorylated in its N-terminal domain (Papavassilliou et al., 1995, Rahmsdorf, 1996). More specifically, phosphorylation of serine 63 and 73 residues and threonines 91 and 93 is evident in response to numerous extracellular stimuli (Franklin et al., 1992; Natoli et al., 1994; Papavassilliou et al., 1995; Radler-Pohl et al., 1993). Phosphorylation of these amino-terminal sites is mediated by one of three mitogen-activated protein kinases (MAPKs). Erk1/Erk2 (p42/p44) were the first proteins which demonstrated the ability to activate c-Jun (Chen et al., 1992). Subsequently, the c-Jun n-terminal kinase (JNK), also termed stress-activated protein kinase (SAPK), and p38 kinase have both been shown to activate c-Jun via N-terminal phosphorylation (Dérijard et al., 1992; Rouse et al., 1994). Similar domains (Thr-N-Tyr) exist within each of these kinases and phosphorylation within this region is essential for the activation of kinase activity. In addition, although extracellular growth factors that act through the epidermal growth factor receptor and Ras proteins will activate all three pathways, there appears to be stimulus-specific effects on each kinase. For example, anisomycin has a modest effect on Erk1/Erk2 while exhibiting a very strong activation of SAPK/JNK and/or p38 (Cano et al., 1994, Cano and Mahadevan, 1995). This specificity of action is likely a result of events that occur upstream of each kinase, and

demonstrates both the complex organization of cell signalling and the cross-talk that exists between pathways.

1.3.2. Sp1

Sp1 is an ubiquitous zinc finger transcription factor that binds to GC-rich regions within the promoter of many cellular genes (Berg, 1992; Jones et al., 1987; Kadonaga et al., 1987; Phillipsen and Suske, 1999). Named for the method of its original method of purification (Sepharose and phosphocellulose columns; Kadonaga et al., 1987), Sp1 is a member of a class of GC-rich binding proteins which includes Sp2, Sp3, Sp4 and Egr-1/zif268 (Berg, 1992). The critical structural differences among these proteins occur within the zinc finger DNA-binding regions and confer the ability for each to bind a different consensus sequence (Berg, 1992). The Sp1 DNA-binding domain consists of an 81 amino acid sequence that recognizes the sequence 5′-GGGGCGGGGC-3′ (Kriwacki et al., 1992). It is known that other members of the Sp family can bind this sequence, albeit at a much lower affinity (Hagen et al., 1992). Contained within the DNA-binding domain are three zinc fingers motifs, within which are critical amino acid sequences thought to interact with the DNA. These sequences are Lys-His-Ala within the first zinc finger, Arg-Glu-Arg within the second and Arg-His-Lys within the third (Phillipsen and Suske, 1999). These regions are highly conserved among many of the members of the Sp family (except Sp2), which indicates the importance of this region in Sp1 function. The ubiquitous expression of Sp1 suggests that it is a vital factor in the regulation of gene expression, and this protein has been implicated in cell cycle regulation, chromatin remodelling and other important cellular

functions (Birnbaum et al., 1995; Ellis et al., 1996; Karlseder et al., 1996). Surprisingly, Sp1 null mutant cells have been developed and they appear to grow and differentiate normally *in vitro* (Marin et al., 1997). However, the generation of Sp1 knockout mice has demonstrated that Sp1 null embryos demonstrate slow growth and all die by day 11 of gestation. These embryos exhibit a wide range of abnormalities at day 11, appearing as an undifferentiated mass of cells up to what appears to be a normal embryo possessing a heart, eyes and other hallmarks that are characteristic of embryonic development (Marin et al., 1997). This phenotype likely suggests that Sp1 plays a more critical role in cell maintenance compared to cell differentiation. However the vast differences in phenotype may also indicate that other members of the Sp1 family can help to combat deficiencies in Sp1 content during differentiation.

Sp1 has been shown to play a role in the transcription of some TATA-less promoters (Smale, 1997) further suggesting the involvement of Sp1 in cell maintenance. In addition, this makes Sp1 a likely target for many transcription factors and other proteins capable of regulating transcriptional activation. It has been recently shown that Egr-1, an immediate early gene that responds to numerous stimuli (Abu-shakra et al., 1993; Michel et al., 1994), can inhibit Sp1 activity through a physical displacement of the protein from the promoter (Huang et al., 1997; Silverman et al., 1997). Furthermore, histone deacetylase, a protein seemingly unrelated to Sp1, has been shown to repress Sp1 function by a direct protein-protein interaction (Doetzlhofer et al., 1999). Thus, it appears that Sp1 is an important factor whose regulation is paramount in the expression of many cellular genes. It is known that

many of the genes encoding mitochondrial protein contain Sp1 sites within their promoters (Lenka et al., 1998), and it has recently been shown that overexpression of Sp1 can indeed activate the expression of these genes (Zaid et al., 1999). Consequently, it is evident that Sp1 plays a role in the maintenance of mitochondrial protein expression, and it is likely that Sp1 activation is a pivotal step in the regulation of mitochondrial biogenesis.

1.4. mRNA Stability

The degradation of mRNAs within the cytosol is a complex process that provides the cell with a non-transcriptional mechanism for regulating the levels of cellular transcripts. In addition, it has been shown that mRNA stability is altered in response to numerous cellular perturbations (Ross, 1995). Most mRNAs are degraded within the cytosol by two classes of cellular nucleases. Exoribonucleases degrade mRNAs from the 3'end while endoribonucleases cleave mRNAs at sites within their sequences (Binder et al., 1989). Endoribonuclease cleavage is usually aided by a protein bound to the mRNA and the resultant fragments are degraded by exoribonucleases within the cytosol (Day and Tuite, 1998, Ross, 1996).

1.4.1. Poly (A) Tail

Most eukaryotic mRNAs have a poly A tail of ≈ 200 nucleotides added to their 3' end. This *cis* element can act to confer mRNA stability, since an inverse relationship exists between poly (A) tail length and mRNA degradation rate (Shapiro et al., 1988). The addition of these nucleotides may act to delay the access of exoribonucleases to the coding region, thereby prolonging message half-life. It has been shown that deadenylation is the first step

in the decay of certain mRNAs (Lieberman et al., 1992; Shyu et al., 1991) which suggests that the presence of a poly (A) tail may act to increase mRNA stability. In addition, there are proteins which bind within this region, termed poly (A) binding proteins, which act to prevent nuclease degradation of the transcript (Bernstein et al., 1989).

1.4.2. Poly (A) Binding Protein

The existence of a protein which binds to the poly (A) tail of mRNA within the cytosol has been known for some time (Blobel, 1973). This poly (A) binding protein (PABP) likely acts to prevent or delay poly (A) tail removal and the subsequent degradation of the mRNA coding region (Ross, 1995). The discovery of PABP may help to explain the inverse relationship between poly (A) tail length and mRNA half-life (Shapiro et al., 1988). Although the longer poly (A) tail may just delay access of exoribonucleases to the coding region, which will act to maintain message integrity, it is possible that as the length of the poly (A) tail increases more PABP molecules can bind to the mRNA, thereby preventing its degradation.

1.4.3. AU-rich Regions

It is important to note that not all of the elements responsible for regulating mRNA stability act to increase half-life. Many short-lived mRNAs contain an AU-rich element (ARE) within their 3′-untranslated region (UTR; Ross, 1995). The destabilizng characteristics of the ARE have been well documented and were first illustrated by Shaw and Kamen (Shaw and Kamen, 1986). When the 3′-UTR from granulocyte macrophage colony stimulating factor (GM-CSF) was inserted at the end of the coding region of β-globin

mRNA, the normally stable β-globin mRNA displayed decay rates similar to the unstable GM-CSF mRNA (Shaw and Kamen, 1986). This mRNA destabilizing effect of the ARE has been demonstrated numerous times in since. Some of the most extensively studied ARE-containing mRNAs are those encoding immediate early genes, which usually code for proteins involved in the transcription of nuclear genes. The 3′-UTR of c-fos contains several AUUUA pentamers and an adjacent U-rich region. It is thought that the ARE facilitates degradation of the mRNA coding region while the U–rich region promotes deadenylation and accentuates the destabilizing effect of the ARE (Alberta et al., 1994; Chen and Shyu, 1994). However, not all AREs are created equal and these regions have been shown to differentially destabilize mRNAs (Ross, 1995). This is evident by the fact that cytochrome c, a very stable mRNA (Freyssenet et al., 1999b), contains an AUUUA sequence within it's 3′-UTR. Contractile activity has been shown to both increase cytochrome c mRNA stability (Freyssenet et al., 1999a) and decrease RNA-protein interactions within the 3′-UTR (Yan et al., 1996) in skeletal muscle. In addition, recent reports suggest that AUF1, an ARE-binding protein (see below), binds within this region and this binding is reduced in response to contractile activity (McClure and Hamilton, 1999). Thus, it appears that the ARE located within the 3′-UTR is involved in the regulation of cytochrome c mRNA stability in skeletal muscle.

1.4.4. ARE Binding Proteins

It is clear that the effects of the ARE are mediated by cytosolic proteins which bind to that region, since cyclohexamide treatment to inhibit protein synthesis stabilizes ARE-

containing mRNAs (Brewer and Ross, 1989). In addition, degradation of these mRNAs will only proceed in the presence of cytosolic proteins (Wilson and Brewer, 1999). The best defined protein of this type is AUF1 (Wilson and Brewer, 1999). AUF1 is a 37 kDa protein that contains two unique RNA recognition motifs (RRM) which have also been identified in other RNA-binding proteins (Wilson and Brewer, 1999; Zhang et al., 1993). AUF1 has been localized to chromosomes 4 and X, and this gene codes for multiple isoforms of AUF1 which are generated through alternative splicing of the pre-mRNA (Wagner et al., 1996; Wilson and Brewer, 1999). However, it remains unclear as to exactly how AUF1 exerts its function. It has been shown that isolated AUF1 binds to RNA but has no affect on mRNA turnover *in vitro* (Wilson and Brewer, 1999), which suggests that AUF1-mediated mRNA decay occurs through a complex of proteins that includes AUF1. In solution, AUF1 exists as a homodimer and likely binds to the ARE as a hexamer (DeMaria et al., 1997). In addition, alterations in AUF1 expression have been shown to mirror changes in the destabilization of β-androgen receptor and GM-CSF mRNAs (Buzby et al., 1996; Pende et al., 1996).

In addition to AUF1 there are other proteins which bind to AREs within the 3′-UTR of mRNAs, although they are not as well understood. There are mammalian proteins which have sequence homology to the *Drosophila* *E*mbryonic *L*ethal *A*bnormal *V*ision (elav) proteins. These elav-like proteins contain 3 RRMs, 2 of which are located in the N-terminal region with the other found in the C-terminus (Wilson and Brewer, 1999). The N-terminal

RRMs bind to the ARE while the C-terminal RRM has been shown to bind to poly (A) sequences (Chung et al., 1996; Ma et al., 1997). The elav-like proteins are thought to bring the poly (A) tail into close proximity to the ARE and, unlike AUF1, have been shown to stabilize glucose transporter 1 mRNA in adipocytes (Jain et al., 1997). The binding of elav-like proteins may act to prevent ribonucleolytic cleavage of the mRNA or may prevent the binding of destabilizing factors (i.e. AUF1) to the ARE. Thus, the same *cis*-element (ARE) can initiate opposite effects depending on the protein binding within that region.

Interestingly, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has also demonstrated the ability to bind tc the ARE (Mogernegg et al., 1986; Nagy and Rigby, 1995), and this binding activity has been attributed to the NAD^+-binding region of the enzyme (Nagy and Rigby, 1995). AUH, a protein isolated from brain tissue, demonstrates a hyrdratase activity and has been shown to bind to the ARE (Nakagawa et al., 1995). This RNA binding activity has been localised to a 20 amino acid sequence which has no homology to other known RNA-biding motifs (Nakagawa and Moroni, 1997). However, the functional significance of the RNA-binding capabilities of either of these enzymes has yet to be established.

1.4.5. Stem-loop Structures

Many different mRNAs possess a stem-loop structure in their UTR. The effects of these stem-loop structures on mRNA stability varies, and a few examples will be discussed below.

Histone mRNA lacks a poly(A) tail but possesses a 6 bp stem-4 bp loop structure

located with the first 30 nucleotides of its 3′-UTR which appears to be involved in regulating mRNA stability (Ross, 1995). This is demonstrated by the observation that when the coding region of the normally stable globin mRNA (Russel et al., 1997) is attached to the 3′-UTR of histone mRNA, the globin mRNA displays the decay characteristics of wild-type histone mRNA (Luscher et al., 1985; Pandey and Marzluff, 1987). Histone mRNA is normally found in very low abundance during the G_1 phase of the cell cycle but the levels of histone mRNA rise sharply during S phase. During S phase the half-life of histone mRNA is 40 minutes (Ross,1995) and in late S phase this half-life falls to 10 minutes (Harris et al., 1991; Heintz et al, 1983). If the region 3′ of the histone stem-loop in the UTR is elongated and polyadenylated, mRNA levels are not properly regulated and likely not destabilized at the appropriate time points (Levine et al., 1987). Thus, the location of the stem-loop structure is important in destabilizing histone mRNA and this effect is likely mediated by an unknown stem-loop binding protein.

Histone mRNAs are not the sole proprietors of mRNA stem-loop structures which alter the expression of cellular mRNAs or proteins. The mRNAs encoding the transferrin receptor and ferritin, both of which are involved in cellular iron homeostasis, contain stem-loop structures termed iron-responsive elements (IREs) and are regulated posttranscriptionally (Klausner et al., 1993; Rouault and Klausner 1997). The transferrin receptor imports iron into the cell while ferritin is responsible for intracellular iron storage. When there is a need for iron within the cell ferritin levels are reduced while there is an increase in transferrin receptor content. This response is mediated by corresponding changes

in the levels of the mRNAs which code for these proteins (Harford and Klausner, 1990), and is mediated by the following mechanism. Two cellular proteins, known as the iron regulatory proteins (IRPs) 1 and 2, regulate the expression of both ferritin and the transferrin receptor by binding to the stem-loop structures located within their mRNAs. Ferritin mRNA contains its IRE within the 5′ region. During conditions of low cellular iron levels, IRPs bind to this region and act to inhibit translation of the ferritin mRNA. This effectively reduces the levels of ferritin within the cell, thereby acting to reduce iron storage which results in an elevation in cellular free iron levels (Hentze et al., 1987). In contrast, the 3′ IRE of the transferrin receptor mRNA acts to stabilize the mRNA via interactions with IRPs (Casey et al., 1988; Owen and Kühn, 1987). During conditions of iron depletion IRPs bind to the 3′ IRE within the transferrin receptor mRNA and results in a 20- to 30-fold increase in message stability. This results in elevations in the levels of the transferrin receptor, thus increasing cell iron uptake capacity (Rouault and Klausner, 1997). It has also been demonstrated that if either the 5′ stem-loop of ferritin mRNA or the 3′IRE of the transferrin receptor mRNA is placed on a non-related reporter mRNA, its expression is subsequently regulated by cellular iron levels (Casey et al., 1988; Hentze et al., 1987). It is unclear as to whether the actions of the IRPs are dependent on the iron content of the cell, or whether the levels of cellular free heme are responsible for mediating IRP activity and the subsequent regulation of iron levels within the cell.

1.4.6. 5′ Untranslated Region and mRNA Cap

The significance of the 5′-UTR in mediating mRNA stability remains relatively

undefined (Ross, 1995). It has been reported that alterations in mRNA stability can be brought about by introducing a translation-inhibiting stem-loop in the 5′-UTR (Aharon and Schneider, 1993). In addition, the unstable mRNA encoding c-myc has also demonstrated stabilization by lengthening of the 5′-UTR in a translation-independent manner (Bonnieu et al., 1988). If this 5′-UTR is lengthened by only 40 nucleotides mRNA stability is unaffected while a 600 bp insertion stabilizes the mRNA 5-fold (Bonnieu et al., 1988). Thus, there may be a protein which is capable of interacting within the extended 600 nucleotide 5′-UTR that cannot bind to the shorter sequence.

One important structure that is found on the 5′ end of nuclear-encoded mRNAs is the 7-methylguanosine cap that is co-transcriptionally added to the mRNA (Lehninger et al., 1995; Salditt-Georgieff et al., 1980). Capped mRNAs are known to be at least fourfold more stable than their uncapped counterparts (Peltz et al., 1987) and suggests that decapping may represent an important step in mRNA degradation.

2. MITOCHONDRIAL BIOGENESIS

The mitochondrion is the cellular organelle which is responsible for the provision of ATP through the aerobic metabolism of substrates (Lehninger et al., 1993). Mitochondrial content varies among tissues within the body and is related, at least in part, to the metabolic activity of the tissue. For example, in vertebrates cardiac muscle has a much higher volume of mitochondria per cell than does skeletal muscle which correlates with a higher, continuous contractile activity in heart compared to the voluntary, intermittent contraction pattern evident in skeletal muscle recruitment (Williams, 1986a). Thus, it is apparent that the

reliance of a tissue on aerobic metabolism dictates the level of expression of mitochondrial proteins. The mitochondrion possesses its own circular double stranded DNA (mtDNA) that is similar to bacterial DNA (Attardi and Schatz, 1988). The entire human mitochondrial genome has been sequenced and is comprised of 16,569 base pairs (b.p.; Anderson et al., 1984) which accounts for less than 1% of total cellular DNA. The mitochondrial genome encodes 13 mRNAs, 22 tRNAs and 2 rRNAs, which fail to account for the large number of proteins located within the mitochondrion. Thus, the majority of mitochondrial proteins must be encoded within the nuclear genome. In addition, it seems likely that there is communication between the nucleus and mitochondrion to coordinate the levels of protein expression during both adaptive and non-adaptive conditions (Scarpulla, 1997). However, the nature of this nucleo-mitochondrial communication is relatively undefined in mammals and remains largely speculative at this point (Grivell, 1995).

During organelle assembly and regular maintenance of intra-mitochondrial protein levels, the genes encoded within the mtDNA must be expressed. To date, only a single nuclear-encoded protein, termed mitochondrial transcription factor A (mtTFA; Clayton, 1984; Clayton, 1991), has been identified and shown to transcribe all of the elements encoded within the mtDNA. Transcription proceeds from the D-loop region and both the heavy and light strands are transcribed as a single strand which is subsequently processed to yield the appropriate mRNAs, rRNAs and tRNAs (Attardi and Schatz, 1989). Like their nuclear counterparts, mitochondrial mRNAs are poly-adenylated, however they lack a 5′-methyl guanosine cap (Anderson et al., 1981). In addition, tissue mitochondrial enzyme

activity appears to be related to mtDNA copy number (Williams, 1986a). Thus, the maintenance of mitochondrial protein levels appear to depend largely on mtDNA replication. Activity-induced elevations in mtDNA content have been shown to parallel changes in both mitochondrial enzyme activity and mRNA level (Annex and Williams, 1990) and this represents a unique aspect in the regulation of mitochondrial genes during organelle biogenesis. During non-proliferating conditions in adult skeletal muscle, nuclear DNA does not replicate thereby conferring a reliance on transcription and mRNA stability in order to bring about changes in cellular mRNA levels.

2.1. Skeletal Muscle

Skeletal muscle is a phenotypically dynamic tissue that responds to chronic contractile activity by altering its protein profile. There are numerous ways to induce mitochondrial biogenesis in skeletal muscle and the information provided below will encompass results from some of these different models.

2.1.1. Mitochondrial Enzymes

Increases in the activities of many mitochondrial enzymes occur in skeletal muscle in response to elevations in contractile activity elicited by both endurance exercise and chronic stimulation (Booth and Thomason, 1991; Essig, 1996; Pette and Vrbová, 1992). Increases in the activities of certain mitochondrial enzymes (e.g. cytochrome c oxidase) parallel increases in content of mitochondria within the cell (Reichmann et al., 1985) and may serve as accurate markers of mitochondrial biogenesis. In addition, chronic stimulation induces elevations in the activity of nuclear-encoded enzymes (i.e. citrate synthase, Williams

et al., 1986b) that are similar to those observed for holoenzymes such as cytochrome c oxidase, which contain nuclear- and mitochondrially-encoded subunits (Connor and Hood, unpublished observation). This co-ordinated adaptation of enzyme activity suggests some form of communication between the nucleus and the mitochondrion, and that activity-induced organelle biogenesis is a tightly coordinated, yet poorly understood phenomenon.

2.1.2. Gene Expression

With the relatively recent elucidation of the DNA sequences of many cellular genes, much of the work examining activity-induced skeletal muscle mitochondrial biogenesis over the last 10-15 years has been directed at determining the regulation of the expression of specific genes of interest. In contrast, much of the earlier investigations on the effects of contractile activity on skeletal muscle phenotype set out to define important adaptations in muscle physiology and function by measuring contractile properties (Salmons and Vrbová, 1969; Pette and Vrbová, 1973), blood flow (Hilton et al., 1970; Hudlická et al., 1977) and enzyme activities (Holloszy, 1967; Pette et al., 1975; Pette et al., 1973). These initial findings demonstrated that the primary physiological response of skeletal muscle to chronic contractile activity (i.e. endurance exercise) was increased muscle performance and resistance to fatigue. In addition, a vast number of subsequent studies have provided a better understanding of how the cell translates an increased contractile activity into adaptations in muscle phenotype. The more recent use of molecular biological techniques for the investigation of activity-induced effects on muscle gene expression have provided valuable insight into the mechanisms which regulate these mitochondrial adaptations (Freyssenet et

al., 1999a; Yan et al., 1996). The *in vivo* activity model represents the current standard method of investigation into these adaptations and has helped to define the process of activity-induced mitochondrial biogenesis. However, this technique does not allow for the precise cellular manipulation that is necessary to fully define the mechanisms that are directly responsible for the observed alterations in muscle phenotype.

The nature of the adaptation of muscle to increased contractile activity has been well characterized *in vivo*. Chronic elevations in muscle activity have been shown to induce increases in the levels of numerous mitochondrial proteins and their associated mRNAs (Freyssenet et al., 1999a; Hood et al., 1989; Williams et al., 1986b; Yan et al., 1996). In addition, these elevations are correlated with elevations in mtDNA copy number which are brought about by mtDNA replication (Annex and Williams, 1990). This characteristic is unique to mitochondrial gene expression and provides a regulatable process which is absent in the expression of nuclear genes encoding mitochondrial proteins. Furthermore, it illustrates the complexities involved in the coordination of organelle biogenesis. Chronic stimulation of rat tibialis anterior muscle is effective in elevating the levels of many mitochondrial proteins (Essig, 1996; Pette and Vrbová, 1992) however, there appears to be a wide range in the extent of adaptation. Steady-state cytochrome c protein levels are elevated approximately 2-fold after either 9 days of continuous (Yan et al., 1996) or 5 days of intermittent (3 hrs/day) stimulation (10 Hz; Freyssenet et al., 1999a), yet after a similar time period (10 days) the levels of mtHSP70 and HSP60 are elevated by 9.3- and 3.6-fold, respectively (Ornatsky et al., 1995). Interestingly, the adaptation in cytochrome c expression

occurs more rapidly in response to intermittent than to continuous elevations in contractile activity. This suggests that events occurring during the recovery from exercise act to accelerate adaptations in muscle phenotype, a phenomenon that has been alluded to previously (Takahashi et al., 1993). Thus, although there appears to be a coordinated organelle adaptation to contractile activity, the extent of the response appears to vary depending on the protein of interest and the delivery of the stimulus.

The response of mitochondrial protein expression to contractile activity often takes many days and is likely a result of earlier events which occur relatively rapidly after the onset of stimulation, leading to adaptations in the expression of associated mRNAs and proteins. In addition, the regulation of organelle assembly appears to depend on changes in mRNA expression, since alterations in protein levels and enzyme activity are often evident either in parallel with or subsequent to adaptations in mRNA expression (Freyssenet et al., 1999a; Hood et al., 1989; Seedorf et al., 1986; Williams et al., 1986b; Yan et al., 1996). Since steady-state mRNA levels are a result of the equilibrium that exists between gene transcription and mRNA stability, contractile activity must initiate alterations in one or both of these processes. The mRNAs encoding mitochondrial proteins turn over at very low rates (Connor et al., 1996) making investigation into the transcription and degradation of these mRNAs *in vivo* difficult using many of the existing methodologies. It has been recently reported using nuclear run-on analyses that the inducible HSP70 (cytosolic) is transcriptionally up-regulated following 24 hrs of contractile activity (Neufer et al., 1996). Although HSP70 is not a mitochondrial protein, it is involved in the chaperoning of proteins

to the mitochondrion for import to the organelle (Schatz, 1996). Thus, this was one of the first reports of an activity-induced transcriptional activation of a protein that is involved in skeletal muscle mitochondrial biogenesis, and suggested that the use of nuclear run-on may prove useful for investigation into the transcriptional regulation of genes involved in organelle biogenesis. However, this technique often involves the use of large amounts of fresh tissue which is not ideal for use in many of the animal models used to investigate muscle mitochondrial biogenesis which often employ smaller mammals (e.g. rats). In addition, subsequent attempts in our laboratory (Connor and Hood, unpublished observations) have failed to yield consistently reliable results in rat skeletal muscle, making nuclear run-on a less than ideal technique. Thus, the use of other methods or models may be necessary to investigate mRNA turnover in skeletal muscle in response to contractile activity. There have been significant advances towards utilizing more effective techniques to investigate this process and it has recently been shown that both cytochrome c mRNA transcription and stability are increased following contractile activity as measured using direct gene injection and *in vitro* mRNA decay assays, respectively (Freyssenet et al., 1999a). It is known that reporter DNA that is directly injected into skeletal muscle is taken up and expressed at detectable levels (Davis and Jasmin, 1993; Thomason and Booth; 1990; Wolff et al., 1990). This type of analysis allows the measurement of gene transcription *in vivo* while utilizing relatively small amounts of tissue to do so. In addition, *in vitro* mRNA decay assays which utilize cytosolic extracts to measure message degradation have recently been shown to be effective in evaluating the effects of contractile activity on the turnover of

mRNAs which encode mitochondrial proteins. However, although these techniques have proven effective for examining the regulation of stable mRNAs, they do not provide a means by which to improve the understanding of the signalling pathways which are responsible for the initiation of activity-induced muscle mitochondrial biogenesis. It has been recently established that acute contractile activity increases the activities of the mitogen activated protein kinase, c-jun n-terminal kinase and p38 kinase pathways in skeletal muscle (Aronson et al., 1997; Goodyear et al., 1996; Hayashi et al., 1999). However, these observations are primarily descriptive and do not explain which elements of muscle contraction are responsible for these adaptations. For example, membrane depolarization, Ca^{2+}mobilization, cross-bridge cycling and alterations in energy metabolism are events which occur during muscle contraction. Each of these are known to initiate intracellular signalling cascades (Biswas et al., 1999; Eble et al., 1998; Fields et al., 1997; Hardingham et al., 1997; Schmidt, 1995) which could ultimately lead to altered gene expression. Until each of these events can be systematically disrupted and the subsequent consequences evaluated, the essential components of muscle contraction that elicit phenotype adaptations will remain unknown. Thus, the development of a contracting muscle cell culture model may provide an opportunity to fully define the events which are responsible for the process of activity-induced skeletal muscle mitochondrial biogenesis. In addition, manipulation of the intracellular milieu is possible in culture by the forced expression of specific proteins and through the use of the numerous cellular inhibitors that exist on the market. This type of control over the cellular environment will allow for more precise experimental control and

may lead to the definition of the mechanisms responsible for activity-induced mitochondrial biogenesis. Contracting cell culture models have been used for the study of mitochondrial proteins in primary cardiac myocytes (Xia et al., 1997, Xia et al., 1998), and should be a feasible option for use in skeletal muscle.

2.2. Other Tissues

Mitochondrial biogenesis is not confined to skeletal muscle, and is a vital component of cellular survival in numerous tissues throughout the body. I will briefly summarize this process as it relates to nerve tissue and cardiac muscle.

Mitochondria are present in neurons and, as would be expected, appear to be localized to areas requiring high amounts of energy. CYTOX activity in gray matter is much higher than in white matter (Hevner et al., 1995), suggesting a greater energy demand in non-myelinated tissues. In addition, many deleterious neurological diseases, such as Alzheimer's, Parkinson's disease, Amyelinating lateral sclerosis (ALS) and multiple sclerosis are associated with decreased mitochondrial enzyme activities. This illustrates a vital role for the organelle in the maintenance of neuronal function (Heals et al., 1999; Tatton and Olanow; 1999; Wong-Riley et al., 1997). Furthermore, the reduced capacity of the mitochondrion to provide energy for the central nervous system (CNS) may represent a possible explanation for the severe decrements in CNS function that are associated with these afflictions. In addition, improvement in mitochondrial function may represent a potential avenue for the development of successful treatments for these debilitating neurological disorders. Mitochondria have recently been shown to play a pivotal role in apoptosis, and

reductions in mitochondrial enzyme activities occur coincident with neuronal cell death (Martin et al., 1999). Thus, it appears that mitochondria are also vital for the maintenance of nerve cell integrity and survival. Interestingly, there appears to be some ambiguity in the response of neurons to changes in activity. For example, blockade of optic nerve action potentials with tetrodotoxin (TTX) selectively alters the content of mitochondrial proteins in the retina (Liu and Wong-Riley, 1995; Wong-Riley et al., 1998). TTX blocks the light-evoked responses of the retina and resulted in decreases CYTOX activity and associated reductions in the levels of both nuclear- and mitochondrially-encoded CYTOX subunit mRNAs and proteins. In addition to the cells of the retina, these adaptations occur in the primary visual cortex, suggesting that activity-induced adaptations are capable of being transmitted from the initial site of application (retina) to areas downstream of the treatment (visual centre). This type of adaptation also occurs in skeletal muscle when motor nerve activity is disrupted with TTX, and suggests an universality of energy-dependent regulation of tissue mitochondrial content. Surprisingly, reductions in the levels of nuclear-encoded mitochondrial CYTOX protein subunits are greater in magnitude and earlier in onset than those for mitochondrially-encoded subunits. This contradicts the idea that regulation of CYTOX holoenzyme activity depends on the catalytic subunits and resides within the nuclear-encoded subunits which are thought mainly to modulate enzyme function by allosteric modification of the mitochondrially-encoded subunits (Lenka et al., 1998). Hypoxia has been shown to decrease CYTOX activity by 20-30% while high levels of neural activity were maintained (LaManna et al., 1996). This maintenance of function in the face

of compromised mitochondrial content does not often occur in skeletal muscle, thereby emphasizing the existence of tissue- and stimulus-specific mechanisms of mitochondrial adaptation. During development, the levels of CYTOX subunit IV and HSP60 increase post-natally in a similar fashion, suggesting co-ordinated regulation of nuclear-encoded mitochondrial genes throughout development (D'Souza and Brown, 1998). It has also been proposed that mitochondria play a role in establishing neuronal polarity, likely by controlling local Ca^{2+} and/or energy gradients (Mattson, 1999). These observations indicate the importance of mitochondrial biogenesis in neuron development and suggest a coordinated regulation of the nuclear genes encoding mitochondrial proteins during this process.

Cardiac muscle is also a phenotypically dynamic tissue that responds to alterations in external stressors (Sadoshima and Izumo, 1997; Xia et al., 1997; Xia et al., 1998). During development, the heart undergoes mitochondrial biogenesis as indicated by progressive increases in CYTOX enzyme activity and the levels of cytochrome c mRNA and protein levels (Stevens et al., 1995). Interestingly, it has been proposed that gene expression in the heart is regulated, in part, by endocrine and paracrine effects (Sadoshima and Izumo, 1997). Indeed, cardiac mitochondrial biogenesis occurs in response to pressure-induced cardiac hypertrophy (Nishio et al., 1995a, Nishio et al., 1995b). However, this response is attenuated by methimazole treatment, which renders the animal hypothyroid. In addition, pressure overload-induced cardiac hypertrophy only occurs if aortic constriction is conducted above the renal artery in order to stimulate the renin-angiotensin system. Taken together, these observations validate the notion of an endocrine contribution to cardiac mitochondrial

biogenesis. In addition, thyroid hormone-induced cardiac hypertrophy has been shown to be correlated with alterations in the levels of nuclear-encoded mitochondrial proteins which are located in the outer mitochondrial membrane or within the matrix of the organelle (Craig et al., 1998). This observation suggests a coordinated regulation of nuclear genes during mitochondrial biogenesis, a similar pattern to that observed in neurons. Furthermore, changes in CYTOX activity occurred in the absence of changes in CYTOX subunit VIc mRNA, suggesting a role of protein turnover in this response. These data are consistent with those reported for skeletal muscle undergoing mitochondrial biogenesis for a similar time period (Hood et al. 1989) suggesting that some common pathways may be regulating mitochondrial biogenesis in these two striated muscle tissues. The anti-AIDs drug zidovudine (AZT) is a thymidine analog that acts to inhibit HIV viral replication by terminating DNA transcription. However, although this drug is somewhat effective, one harmful side effect is that it interferes with mitochondrial DNA replication, which may confer deleterious side effects on cellular metabolism and ATP formation. AZT treatment has been shown to decrease the levels of the mitochondrially-encoded CYTOX subunit III mRNA and induce a parallel reduction in CYTOX holoenzyme function while having no effect on CYTOX subunit VIc mRNA (Freyssenet et al., 1999c; McCurdy and Kennedy, 1998). These observations are consistent with the idea that CYTOX activity is regulated by the mitochondrially-encoded catalytic subunits (Lenka et al., 1998), and appears to be differ from the pattern evident in nerve tissue. Recent experiments have attempted to examine activity-induced alterations in cytochrome c expression in cardiac myocytes in culture (Xia

et al., 1997, Xia et al., 1998). These studies have shown that CYTOX activity is increased by muscle contraction and the expression of the nuclear-encoded cytochrome c gene is regulated by c-jun and NRF-1. This type of analysis has yet to be conducted in skeletal muscle myocytes, and would represent a major step in understanding the events responsible for contractile activity-induced alterations in gene expression in skeletal muscle.

Thus, it is clear that mitochondrial biogenesis in excitable cells (e.g. neurons, cardiac muscle and skeletal muscle) share some common regulatory responses. Most notably, mitochondrial content appears to be associated with the metabolic activity of the tissue, with increases or decreases in demand resulting in parallel changes in mitochondrial content. However, it is clear that the coordination of this response is extremely complex and the mechanisms by which these adaptations are achieved vary among tissues.

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Rationale for Manuscript # 1

The ramifications of muscle disuse on tissue structure and function are vast and the mechanisms responsible are complex and somewhat poorly understood. Many different models exist to study the effects of reduced muscle activity on skeletal muscle phenotype. However, although these models are very effective in creating conditions of decreased muscle use, they each possess some inherent problems. Denervation renders muscle completely inactive while removing the influence of trophic factors on the muscle. Moreover, this treatment severely impairs the ambulatory pattern of the animal. Treatment with TTX is reversible and keeps the trophic influence of the nerve on the muscle intact, but does not remedy the ambulatory perturbations. Microgravity provides a unique model of decreased muscle use in that both nerve function and limb movement remain intact. However, the response of skeletal muscle to microgravity is complex and remains poorly defined.

This study was designed to gain insight into the response of the genes encoding mitochondrial proteins to microgravity exposure in skeletal and cardiac muscle following spaceflight. Thus, I measured the levels of mitochondrial enzyme activities, protein levels and the corresponding mRNAs in heart and skeletal muscle in order to develop a better understanding of some of the molecular events which characterize the physiological adaptations occurring in these tissues under conditions of microgravity. I was responsible for all of the experimental work in this paper and Dr. Hood served as the principal investigator of the study.

MANUSCRIPT # 1:

Effect of microgravity on the expression of mitochondrial enzymes in rat cardiac and skeletal muscle.

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Effect of microgravity on the expression of mitochondrial enzymes in rat cardiac and skeletal muscle.

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Running title: Effects of microgravity on heart and skeletal muscle

ABSTRACT

The purpose of this study was to examine the expression of nuclear and mitochondrial genes in cardiac and skeletal muscle (triceps brachii) in response to short-duration microgravity exposure. Six adult male rats were exposed to microgravity for six days and were compared to ground-based control animals (n=6). We observed a significant 32% increase in heart malate dehydrogenase (MDH) enzyme activity, which was accompanied by a 62% elevation in heart MDH mRNA levels, following microgravity exposure. Despite modest elevations in the mRNAs encoding subunits III, IV and VIc, as well as a 2.2-fold higher subunit IV protein content following exposure to microgravity, heart cytochrome c oxidase (CYTOX) enzyme activity remained unchanged. In skeletal muscle, MDH expression was unaffected by microgravity, but CYTOX activity was significantly reduced 41% by microgravity, while subunit III, IV and VIc mRNA levels, and subunit IV protein levels were unaltered. Thus, tissue-specific (i.e. heart vs. skeletal muscle) differences exist in the regulation of nuclear-encoded mitochondrial proteins in response to microgravity. In addition, the expression of nuclear-encoded proteins such as CYTOX subunit IV and MDH are differentially regulated within a tissue. Our data also illustrate that the heart undergoes previously unidentified mitochondrial adaptations in response to short-term microgravity conditions, more dramatic than those evident in skeletal muscle. Further studies evaluating the functional consequences of these adaptations in the heart, as well as those designed to measure protein turnover, are warranted in response to microgravity.

Index terms: spaceflight; gene expression; heart; mitochondrial biogenesis

Mammalian skeletal muscle is a phenotypically dynamic tissue capable of altering its metabolic protein profile in response to changes in physiological demand. There are many models of decreased muscle use, such as denervation, immobilization and hindlimb unweighting, which lead to large changes in gene expression (3) and fiber size (1). A typical adaptation evident in the literature is a reduction in the levels of muscle mitochondrial proteins (16, 20). In contrast, the decreased use imposed by exposure to microgravity appears to produce an inconsistent and relatively poorly understood mitochondrial protein adaptation in skeletal muscle (1,19). For example, 3-hydroxyacyl-CoA dehydrogenase (HAD) levels were decreased in the soleus following a 7 day spaceflight (9) however, HAD expression was unaffected in vastus lateralis and vastus intermedius muscles after a 9 day exposure to microgravity (2). In addition, while pyruvate oxidation was unchanged by 9 days of spaceflight, the rate of palmitate oxidation was reduced (2). These data illustrate the need for further investigation into the effects of short-duration microgravity exposure on skeletal muscle metabolic enzymes. Furthermore, few measurements have been made of the mRNAs which encode metabolic enzymes (24), and no studies have compared the expression of specific mitochondrial proteins and their respective mRNA levels. In addition, much of the existing work has been related to skeletal muscle and very little is known about the response of the heart to microgravity. During spaceflight, the effects of gravity on the cardiovascular system are removed, and the blood volume that is normally pooled in the lower limbs in a 1*g* environment is shifted to the thorax, thereby temporarily increasing venous return (10, 25). This shift in blood volume may cause alterations in gene expression via pathways

similar to those utilized during stretch-induced cardiac hypertrophy (21). Thus, the overall purpose of this study was to determine the effects of a short-duration spaceflight on cardiac and skeletal muscle mitochondria at both the protein and mRNA levels of expression. This analysis should help us better understand the regulation of the expression of genes encoding mitochondrial proteins in these tissues when subjected to microgravity.

METHODS

Experimental animals. Adult male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were exposed to microgravity for 6 days (final mass 216 ± 4 g; n=6) aboard the Shuttle Transport System-54 mission. Similar age-matched animals (233 ± 8 g; n=6) served as ground-based controls. All animals were fed and housed similarly, as previously described (5). Portions of the triceps brachii and heart muscles from animals subjected to microgravity were excised and snap frozen in liquid N_2 3-9 hours following the completion of the mission. Portions of the heart and triceps brachii muscles from control animals were removed the following day during a similar time period.

Wet:dry mass ratios. Triceps brachii and heart fragments were pulverized to a powder at the temperature of liquid N_2. Portions of the powders were dried overnight at 70°C to a constant weight, and the ratio of wet-to-dry mass was calculated.

Enzyme analyses. Frozen triceps brachii and heart tissue powders were used to determine cytochrome c oxidase (CYTOX) activity by measuring the oxidation of reduced cytochrome c (11). These tissue powders were also used to measure glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and malate dehydrogenase (MDH) enzyme activities as previously described (18). All of these enzyme activities were determined photometrically (Beckman DU-64) at 30°C.

mRNA measurements. Total RNA was isolated from heart and triceps tissue powders as previously described (6). Ten μg of total RNA were separated on a 1% agarose mini-gel and transferred to a nylon membrane (Hybond-N, Amersham). RNA was fixed to the

membrane with ultraviolet light, and these northern blots were hybridised overnight with ^{32}P-dCTP labelled cDNAs specific for CYTOX subunits III, IV and VIc, and MDH mRNAs as well as 18S rRNA. Blots were washed initially 3 X 10 min with 2X SSC, 0.1% SDS at room temperature. All of the blots were then washed for 15 min at 55°C with 0.1X SSC, 0.1% SDS and then subsequently washed for 15 min at 60°C with 0.1X SSC, 0.1% SDS. The levels of specific mRNAs were quantified following a 1-24 hour exposure using electronic autoradiography (Packard), and 18S rRNA levels were used to correct for uneven loading between lanes.

Western blotting. Proteins from triceps brachii muscle (20 μg; n=6) and heart (15 μg; n=6) were separated on a 15% polyacrylamide gel using one-dimensional SDS/PAGE. Proteins were electrotransferred to nitrocellulose membranes (Hybond-C, Amersham) for subsequent immunoblotting, as done previously (8, 17, 23). Following blocking in 5% skim milk/1.25% horse serum, membranes were incubated with a monoclonal antibody directed against CYTOX subunit IV, at a working dilution of 1:1000 in 1% skim milk. Goat anti-mouse IgG conjugated alkaline phosphatase (1:750 dilution) was used as the secondary antibody. CYTOX subunit IV proteins were visualised using a colour reaction, and the bands were quantified by laser densitometry.

Statistical analyses. Values presented are means ± SEM and statistical significance was determined using Student's t-tests for independent samples. Differences were considered statistically significant at the 0.05 confidence level.

RESULTS

Wet:dry mass ratios. The wet:dry mass ratios were 4.01 ± 0.08 for control triceps brachii, 4.59 ± 0.25 for space triceps brachii, 4.10 ± 0.07 for control heart and 4.11 ± 0.06 for space heart tissues. There were no significant differences among these values.

Protein expression. Six days of microgravity exposure resulted in a significant 32% increase in heart mitochondrial MDH enzyme activity compared to that in control hearts. No significant effect of microgravity on heart CYTOX activity or GAPDH activity was observed (Table 1). Despite the lack of change in CYTOX holoenzyme activity in heart muscle, microgravity induced a 2.2 ± 0.2-fold increase ($n=6$; $p<0.05$) in CYTOX subunit IV protein level compared to the hearts of ground-based control animals (Fig.1). The response of skeletal muscle to microgravity differed from that of cardiac muscle. CYTOX activity in the triceps muscle was significantly decreased by 41% following microgravity exposure compared to the activities found in the triceps of control animals (Table 1). However, MDH enzyme activity in skeletal muscle remained unaffected by the microgravity treatment. In contrast, the activity of the glycolytic enzyme GAPDH was significantly elevated by 58% in the triceps muscle in response to microgravity. No effect of microgravity on CYTOX subunit IV level was observed in skeletal muscle (Fig. 1).

mRNA expression. Total RNA levels in the hearts of animals subject to microgravity (1057 ● 61 μg/g; $n=6$) were not different from those found in the hearts of control animals (1025 ● 126 μg/g; $n=6$). However, changes were observed in the levels of specific mRNAs. In particular, a 62% increase ($p<0.05$) in MDH mRNA following microgravity exposure was

observed in heart muscle (Figs. 2A, 2B). The level of mRNAs encoding CYTOX subunits III, IV and VIc were modestly increased by 23-37%. In contrast to the results observed in heart muscle, the total RNA concentration in skeletal muscle was significantly decreased by 26% following the six day spaceflight, from 916 ± 83 μg/g (n=6) to 682 ± 42 μg/g (n=6) of muscle wet weight. However, there were no corresponding reductions in the levels of the mRNAs encoding MDH, CYTOX subunit III, subunit IV or subunit VIc in the triceps muscle following microgravity exposure (Figs. 3A, 3B).

DISCUSSION

The microgravity environment imposed on an organism during spaceflight provides a unique model for the study of gravitational effects on organ function. Our objective in this study was to determine the extent of the mitochondrial adaptations occurring in both cardiac and skeletal muscle following short-term microgravity exposure. Since changes in mitochondrial content are strongly correlated with performance, at least in skeletal muscle (12), our findings may have implications for muscle performance deficits observed following spaceflight (5, 7). Further, characterization of these changes using multiple enzyme measurements may help to clarify the ambiguities currently evident in the literature with regard to the response of muscle to microgravity (1, 19). With respect to the heart, we are unaware of any study which has documented the extent of metabolic enzyme changes which occur during spaceflight in this tissue. This may provide some insight into potential changes in cardiac functional capacity during microgravity exposure. Our study was uniquely designed to evaluate not only protein measures, but also coincident evaluations of changes in mRNA expression, thereby helping to define precursor (i.e. mRNA)-product (i.e. protein) relationships, and some underlying mechanisms responsible for the changes in protein levels observed.

Tissue-specific differences in the regulation of mitochondrial proteins have been documented previously (6, 22, 26), and our results highlight tissue-specific adaptations to short-term microgravity conditions in two different striated muscle types. Apart from the reduction in CYTOX enzyme activity, gene expression in the triceps muscle remained

relatively unaffected by six days of microgravity. Interestingly, heart muscle displayed significant increases in MDH activity and concomitant augmentations in the levels of MDH mRNA. The other mitochondrial enzyme measured, CYTOX, was not significantly increased, but subunit mRNA levels tended to be higher in the hearts of animals subject to spaceflight. These data identify a previously unrecognized cardiac muscle adaptation to microgravity, in that the heart achieves a greater oxidative capacity subsequent to spaceflight. The mechanisms responsible for inducing this adaptation remain speculative. In humans, it is known that microgravity exposure causes a redistribution of blood volume from the lower limbs to the thorax (25), thereby increasing venous return and end-diastolic volumes in the heart, at least in the early stages (4), despite a concomitant reduction in plasma volume (15). The obligatory stretching of the myocardium which accompanies this altered venous return is known to be a powerful stimulus for the induction of changes in gene expression (21). Thus, alterations in the expression of mitochondrial proteins may also occur in human cardiac muscle exposed to microgravity.

Our data also illustrate the markedly different gene expression responses of mitochondrial proteins to a given physiological pertubation such as microgravity. For example, the 62% increase in MDH mRNA levels was accompanied by a 32% increase in enzyme activity in the hearts of spaceflight animals. These data suggest that, in the heart, the augmented levels of MDH protein may be a result of an increase in cellular mRNA levels due to altered mRNA transcription or stability, matched by a somewhat lesser increase in MDH protein degradation. With regard to CYTOX, a balance may have existed between

the modest increase in subunit mRNAs and subunit protein degradation, leading to unaltered CYTOX enzyme activity. In contrast, a qualitatively different response was observed with CYTOX subunit IV, in which the small increase in mRNA level was accompanied by a much larger increase in subunit IV protein level. This suggests the occurrance of a decrease in subunit IV degradation rate as a result of six days of microgravity, and the results highlight the fact that proteins with similar organellar destinations within the same tissue may be regulated completely differently in response to a physiological pertubation.

The surprisingly large adaptations evident in the heart were not apparent in skeletal muscle, which displayed a much greater resistance to phenotypic alterations in response to microgravity exposure. For example, CYTOX subunit IV mRNA and protein levels were completely unaffected by the treatment in the triceps brachii muscle. MDH mRNA and enzyme activity levels were similarly unchanged. However, it should be noted that since total RNA per g of muscle was significantly reduced in this tissue, this implies that a reduction in both MDH and subunit IV mRNAs did occur when expressed per g of muscle. In order for MDH and subunit IV protein levels to remain constant (Fig. 1, Table 1), an increase in translational efficiency could have occurred, even in the absence of a change in protein degradation rate. With regard to CYTOX, the catalytic properties of the holoenzyme responsible for enzyme activity reside in subunits I, II and III (14). An increase in the translational efficiency of these catalytically-relevant subunits could have been exceeded by an increase in degradation rate, resulting in the decline of enzyme activity observed in the present study. Clearly, further work is needed to corroborate the alterations in degradation

rate of nuclear-encoded mitochondrial proteins which we have interpreted to occur in response to microgravity exposure. Elevated protein degradation rates are expected to occur in view of the muscle atrophy which is observed following a 6 day space flight (5), as well as the enhanced protein turnover reported for non-mitochondrial proteins in skeletal muscle subject to unweighting (3).

Our data also demonstrate that the regulation of a multi-subunit enzyme such as CYTOX is very complex, as evident from the relatively small elevation in subunit IV mRNA, accompanied by the large increase in subunit IV protein levels in the heart, in the absence of changes in CYTOX enzyme activity. It is interesting to note that the mRNAs encoding subunits III, IV and VIc responded similarly to microgravity. This coordinated expression of mRNAs derived from the nuclear and mitochondrial genomes which code for subunits of the same holoenzyme has been previously documented under steady-state conditions (6, 11) and during conditions of altered mitochondrial biogenesis (13, 27).

The microgravity model of decreased muscle use is a valuable one which allows for the elucidation of multiple adaptations occurring in a number of different tissues during and after spaceflight. Our understanding of these adaptations will facilitate the subsequent development of appropriate countermeasures designed to eliminate or modify these changes, thereby alleviating some of the problems which occur upon the return to a 1*g* environment. As extensively discussed by Roy et al. (19) some of the inherent limitations in the design of experiments utilizing microgravity were evident in our study. The relatively low number of animals exposed to microgravity, the long periods of time between the return to 1*g* and tissue

collection (up to 9 hours), as well as the large number of investigators involved in dividing up these tissues, create significant obstacles during data collection and interpretation. Although, we appreciate the logistical constraints involved in exposing animals to a microgravity environment, we also feel that these design shortcomings must be addressed in future work of this kind, if possible.

In summary, the data in the present study illustrate differences in the expression of mitochondrial enzymes in heart and skeletal muscle subject to microgravity in the rat, and they reveal a surprising response in cardiac muscle subject to this condition. Further investigation into the role of protein degradation in the observed adaptations is warranted as a function of microgravity exposure time. In addition, a determination of the physiological consequences of these mitochondrial adaptations is necessary in order to fully understand the cardiac and skeletal muscle responses to spaceflight.

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Fig. 1. The effects of microgravity on CYTOX subunit IV protein expression in skeletal muscle and heart. Proteins from cytosolic extracts from triceps brachii (A; n=6) and heart (B; n=6) muscles were incubated with a monoclonal antibody directed towards CYTOX subunit IV. The levels of this protein in tissues from spaceflight (S) and ground-based control animals (C) were quantified using laser densitometry.

Fig. 2. Effect of microgravity on the expression of mRNAs encoding mitochondrial proteins in heart. Total RNA (10 μg) from the hearts of spaceflight (S) and control (C) animals were probed with cDNAs specific for the mRNAs encoding cytochrome c oxidase (CYTOX) subunit III, subunit IV, subunit VIc and malate dehydrogenase (MDH) as well as the 18S rRNA subunit. Representative blots are depicted in (A) and a graphical summary of all of the samples (n=6) is presented in (B), with values expressed as a percentage of the levels observed in control hearts. Arrows on the left of each autoradiogram indicate the levels of migration of the 28S and 18S rRNAs. *$P<0.05$ compared with control hearts.

Fig. 3. Effect of microgravity on the expression of mRNAs encoding mitochondrial proteins in skeletal muscle. Total RNA (10 μg) from the triceps brachii of spaceflight (S) and control (C) animals were probed with cDNAs specific for the mRNAs encoding cytochrome c oxidase (CYTOX) subunit III, subunit IV, subunit VIc and malate dehydrogenase (MDH), as well as the 18S rRNA subunit. Representative blots are depicted in (A) and a graphical representation of all of the samples (n=6) is presented in (B), with values being expressed as a percentage of the levels observed in control triceps brachii muscle. Arrows on the left of each autoradiogram indicate the levels of migration of the 28S and 18S rRNAs.

Table 1. *Enzyme activities of heart and triceps brachii muscles of control and spaceflight animals*

Enzyme	Control triceps brachii	Space triceps brachii	Control heart	Space heart
MDH	352.4 ± 22.5	379.4 ± 10.8	1462.0 ± 76.0	1923.7* ± 111.8
% of control	-	107.6	-	131.6
CYTOX	11.3 ± 1.8	6.7* ± 0.6	86.6 ± 17.0	96.1 ± 10.4
% of control	-	59.3	-	111.2
GAPDH	1544.9 ± 130.0	2437.0* ± 285.9	621.2 ± 98.7	540.2 ± 14.4
% of control	-	157.5	-	86.8

Enzyme activity values are mean ± SEM and expressed as U/g wet weight; $n = 6$ rats in each group; * $p<0.05$ compared to control.

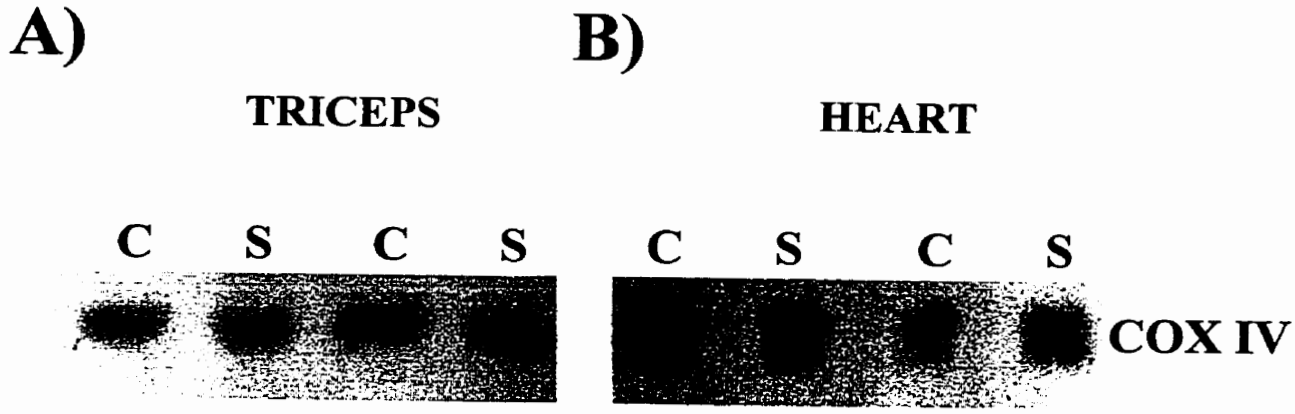

Figure 1

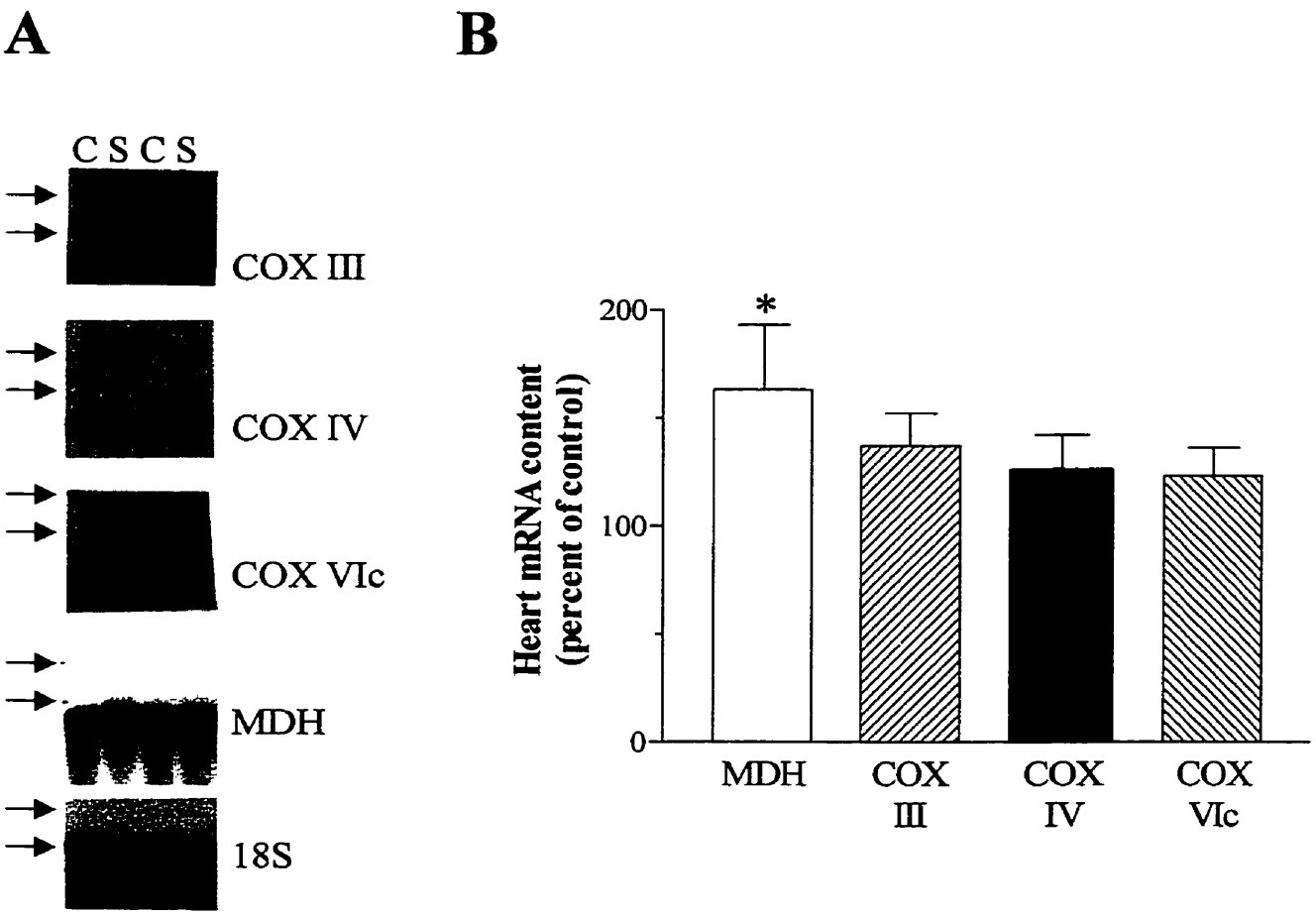
A
C S C S
COX III
COX IV
COX VIc
MDH
18S
B
Heart mRNA content
(percent of control)
200
100
0
*
MDH
COX
III
COX
IV
COX
VIc

Figure 2

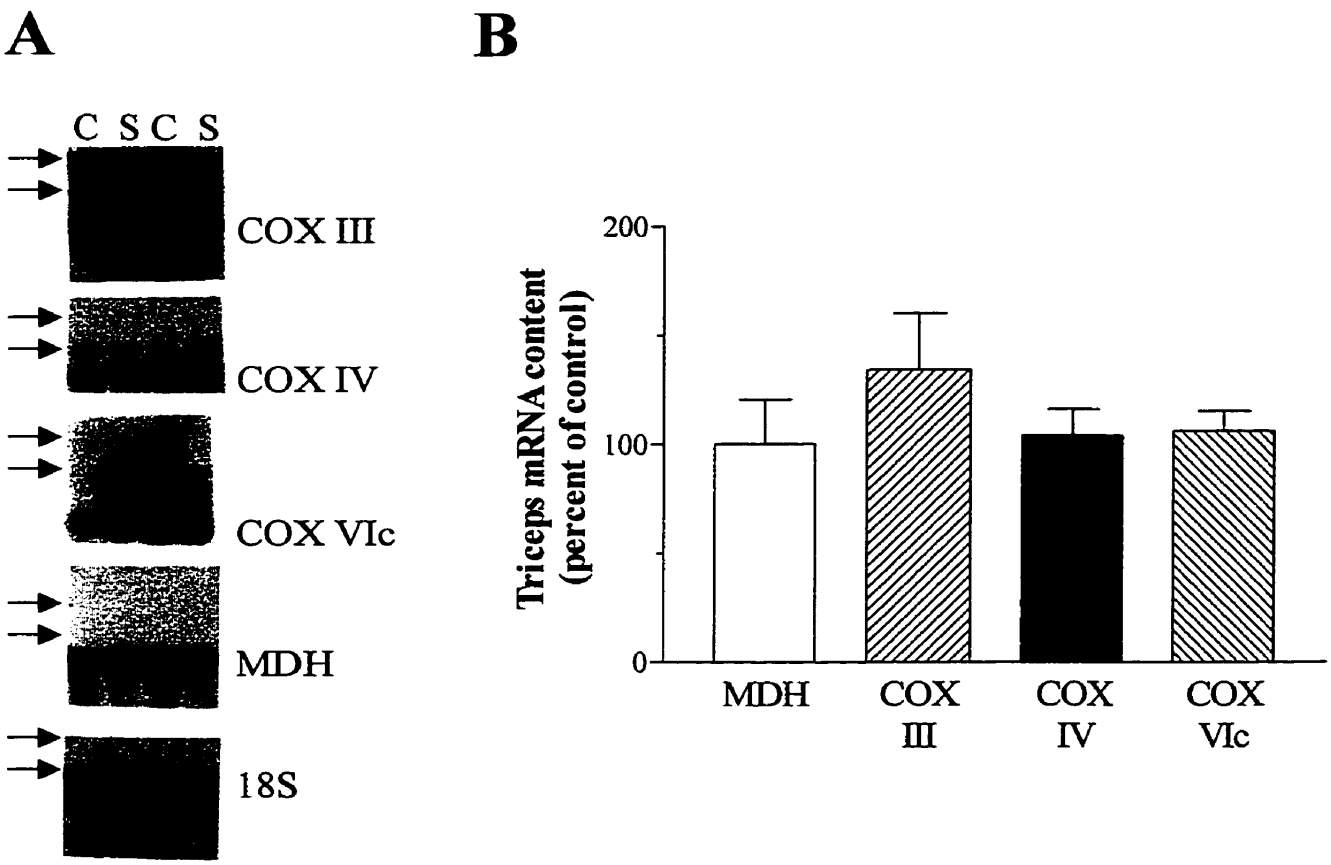
A
C S C S
COX III
COX IV
COX VIc
MDH
18S
B
Triceps mRNA content
(percent of control)
200
100
0
MDH
COX III
COX IV
COX VIc

Figure 3

Rationale for Manuscript # 2

Contractile activity is a potent stimulus for the induction of mitochondrial biogenesis in skeletal muscle. Since only 13 of the proteins contained within the mitochondrion are encoded in the mitochondrial DNA, there is a reliance on the regulation of the expression of nuclear genes for mitochondrial biogenesis to occur. The levels of many of the mRNAs encoding mitochondrial proteins are elevated in response to contractile activity however, the mechanisms behind these adaptations remain unknown. Steady-state levels of a particular nuclear-encoded mRNA result from the balance between its transcription within the nucleus and its degradation (or stability) in the cytosol. Definition of this equilibrium in skeletal muscle has proven difficult due in part to low rates of mRNA turnover. This study was designed in an attempt to measure the transcription of the cytochrome c gene using direct gene injection, and to evaluate mRNA stability by developing an *in vitro* mRNA decay system during mitochondrial biogenesis.

In this study I was responsible for electrode implantation and electrical stimulation of half of the animals and all of the work pertaining to *in vitro* mRNA decay. I was also responsible for a large portion of the manuscript preparation as well as preparation of the figures used therein. Damien Freyssenet was responsible for the measurement of steady-state cytochrome c mRNA and protein levels along with all of the direct gene injection experiments. Mark Takahashi was responsible for half of the electrode implantation surgeries. Dr. Hood served as the principal investigator of the study.

MANUSCRIPT # 2:

Cytochrome c transcriptional activation and mRNA stability during contractile activity in skeletal muscle.

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Cytochrome c transcriptional activation and mRNA stability during contractile activity in skeletal muscle

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Running title: Cytochrome c expression in chronically stimulated muscle.

ABSTRACT

We evaluated contractile activity-induced alterations in cytochrome c transcriptional activation and mRNA stability using unilateral chronic stimulation (10 Hz, 3 hr/day) of the rat tibialis anterior (TA) muscle for 1, 2, 3, 4, 5 and 7 days (n=3-11/group). Transcriptional activation was assessed by direct plasmid DNA injection into the TA using a chloramphenicol acetyltransferase (CAT) reporter gene linked to 326 bp of the cytochrome c promoter. Cytochrome c mRNA in stimulated muscles increased by 1.3- to 1.7-fold above control between 1 and 7 days. Cytochrome c protein was increased after 5 days of stimulation to reach levels which were 1.9-fold higher than control by 7 days. Cytochrome c mRNA stability, determined using an *in vitro* decay assay, was greater in stimulated TA than in control between 2 and 4 days, likely mediated by the induction of a cytosolic factor. In contrast, cytochrome c transcriptional activation was elevated only after 5 days of stimulation when mRNA stability had returned to control levels. Thus, the contractile activity-induced increase in cytochrome c mRNA was due to an early increase in mRNA stability, followed by an elevation in transcriptional activation, leading to an eventual increase in cytochrome c protein levels.

Index terms: cell-free mRNA decay, chronic stimulation, direct gene injection, mitochondrial biogenesis

It is well established that mitochondrial biogenesis can be elicited in skeletal muscle in response to sustained contractile activity (10, 12). Increases in the volume of skeletal muscle mitochondria (9, 20), as well as concomitant elevations in the activity of many mitochondrial enzymes occur in response to increased muscle activity (8, 13). These changes occurring at the protein level of expression are also accompanied by elevations in mRNAs derived from both the nuclear and the mitochondrial genomes (14, 32). Alterations in gene transcription and/or mRNA stability could potentially explain these increases. However, the relative contribution of these processes in response to contractile activity has not been established.

In the present study, we used the expression of cytochrome c as a representative model of the possible adaptations in mRNA turnover which transpire during chronic stimulation. Cytochrome c is a nuclear-encoded mitochondrial protein involved in electron transport between complexes III and IV of the mitochondrial respiratory chain. The sequence of the cytochrome c gene has been extensively studied, and both the coding region (24) and the upstream regulatory elements have been determined (11). In skeletal muscle, cytochrome c mRNA and protein levels are known to be up-regulated in parallel with changes in contractile activity (8, 36). Recently, it has been shown that continuous contractile activity (24 hour/day) induced an alteration in RNA-protein interactions at the 3′ end of the cytochrome c mRNA, suggesting that increases in skeletal muscle cytochrome c mRNA expression may be at least partially mediated by changes in message stability (36). To further investigate this hypothesis, we evaluated both cytochrome c transcriptional activation

and mRNA degradation using a physiologically relevant contraction (3 hrs/day) and recovery period stimulation paradigm. To measure transcriptional activation, we used direct plasmid DNA injection into skeletal muscle. A number of studies have shown that promoter-reporter chimeric gene constructs are successfully taken up and expressed in skeletal muscle (30, 33). Transcriptional activation can be subsequently determined by measuring the expression of the protein encoded by the reporter gene. To evaluate cytochrome c mRNA degradation, we utilized a cell-free mRNA decay system (21) using extracts derived from stimulated and non-stimulated control skeletal muscle. Thus, measurements of both transcriptional activation as well as mRNA stability were used to interpret the time course changes in cytochrome c mRNA expression during chronic contractile activity.

METHODS

Animal care and surgery. Male Sprague-Dawley rats (283 ± 5 g) were housed individually in a temperature-controlled room (21 °C) with a 12h-light/12h-dark cycle, and were allowed food and water *ad libitum*. A smaller group of animals was used for a separate mRNA stability experiment (352 ± 14 g, n=6). Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg body weight) and platinum electrodes (Med-Wire, Leico Industries, New York, NY) were surgically implanted on both sides of the common peroneal nerve of the left hindlimb as previously described (27). This procedure was used to stimulate both the tibialis anterior (TA) and the extensor digitorum longus (EDL) muscles. The contralateral non-stimulated limb served as an internal control. Animals subject to this protocol are able to locomote freely within the cage, and appear to eat and drink in unaffected fashion.

Plasmids. The plasmid constructs (pRC4CATBΔ/-326 and -66) used to assess cytochrome c transcriptional activation were generous gifts of Dr. R. Scarpulla (Northwestern University, Chicago, IL, USA). The -326 construct contains sequences of the rat cytochrome c promoter which include 326 bp upstream of the transcription start site, linked to a chloramphenicol acetyltransferase (CAT) reporter gene. This construct has been previously shown to give full cytochrome c promoter activity in COS-1 cells (11). The -66 construct, which represents the minimal promoter region (11), was used in initial studies for comparison to the -326 promoter. The plasmid directing the synthesis of β-galactosidase (β-gal) under the control of the Rous Sarcoma Virus long terminal repeat (pRSV/β-gal) was also

used with the intent of correcting for DNA transfection efficiency. Plasmid DNA was isolated using an alkaline lysis technique followed by phenol/chloroform extractions. DNA was then dissolved in 0.9% sodium chloride and the DNA concentration was determined at 600 nm using the diphenylamine assay (31). The quality of DNA was assessed by restriction endonuclease digestion and 1% agarose gel electrophoresis.

Direct plasmid injection. One week post-operatively, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg body weight), and a single incision was made in the skin covering the right and left TA muscles. A 27-gauge needle was inserted obliquely 0.1-0.2 cm deep into the TA muscle to inject a combination of pRC4CAT/-326 or -66, and pRSV/β-gal (50 μg each) in a final volume of 100 μl. The incision was then sutured and animals were allowed to recover for 2 days prior to the onset of chronic stimulation.

Stimulation protocol and tissue sampling. Two days following the plasmid DNA injections, TA and EDL muscles were stimulated (10 Hz, 0.1 ms duration, 3 hours/day) for 1, 2, 3, 4, 5 and 7 days (28, 29). On the day following the indicated stimulation time period (21 hours later), animals were anesthetized, TA muscles were removed from both the stimulated and contralateral limbs, weighed and frozen. In a separate experiment designed in part to evaluate the effect of continuous contractile activity on cytochrome c mRNA stability alone, animals were stimulated at 10 Hz for 10 days (24 hours/day), as done previously (27). Six hours after the cessation of stimulation, bilateral TA muscles were removed, frozen and stored. All muscles were then powdered and stored in liquid N_2 until

required for analyses.

RNA analyses. Total RNA was isolated from frozen muscle powders (50-70 mg) as previously described (6). RNA concentration and purity were determined by ultraviolet photometry at 260 and 280 nm, respectively. Total RNA (6 μg) was then separated on a denaturing formaldehyde-1% agarose gel and transferred overnight to a nylon membrane (Hybond N, Amersham, Mississauga, Canada). Cytochrome c and 18S rRNA radiolabelled cDNAs were generated by random primer labelling in the presence of [α-^{32}P]-dCTP (Amersham). Following the removal of unincorporated nucleotides, label incorporation was determined by Cerenkov counting. Blots were prehybridized overnight (42°C) and the membranes were subsequently hybridised overnight at 42°C with the appropriate radiolabelled cDNA probe (2 x 10^6 cpm) as done previously (6). The blots were rinsed with 2 x SSC (0.15 M NaCl/0.030 M sodium citrate), 0.1 % sodium dodecyl sulfate (SDS) and subsequently washed 3 x 10 minutes at room temperature with 2 x SSC, 0.1 % SDS, followed by a 15 minute wash at 50°C in 0.1 x SSC, 0.1% SDS. The blots were quantified by electronic autoradiography (Instantimager, Packard), which measures the total radioactivity in the region of the cytochrome c mRNA. In this case, all three bands corresponding to the cytochrome c mRNA species were quantified. Blots were then corrected for uneven loading with subsequent probing using a cDNA encoding 18S rRNA.

Tissue extraction for protein assays and immunoblotting. Powdered tissues (20-25 mg) were diluted 40-fold (w/v) in 0.1 M KH_2PO_4 buffer (pH 7.2) containing 2 mM EDTA and sonicated (8 x 10 sec) on ice. Samples were then centrifuged for 6 min in a microfuge

at 4°C. The supernates were removed and protein concentration was determined photometrically (2).

SDS gel electrophoresis and immunoblotting. Muscle protein extracts (75 μg) were applied to one dimensional SDS polyacrylamide gels (12.5%, w/v) and electrophoresed overnight. The separated proteins were then electrotransferred to nitrocellulose membranes (Hybond C, Amersham) and incubated overnight with a rabbit anti-rat cytochrome c polyclonal antibody (1:500, 25). The secondary antibody was an alkaline phosphatase-conjugated goat anti-rabbit antibody (1:1000), affording visualisation of the antigen by a standard colour reaction. The immunoblots were then quantified by laser densitometry (28).

CAT and β-galactosidase activities. Muscle powders (100-125 mg) were diluted 3-fold (w/v) in 0.25 M Tris (pH 7.9), subsequently frozen in an ethanol/dry ice bath and thawed at 37°C three times. The resulting homogenates were then centrifuged in a microfuge for 5 minutes at 4°C, and the supernates were used as muscle extracts for CAT and β-gal activities. To assess CAT activity, 10 μl of muscle extract were mixed with 4 μl of 10 mM acetyl-CoA and 8.6 μl (0.215 μCi) of [^{14}C]chloramphenicol (55 mCi/mmol; Amersham). The mixture was adjusted to a final volume of 40 μl with 0.25 M Tris (pH 7.9) and was subsequently incubated at 37°C for 3.5 hours. Chloramphenicol was then extracted from the reaction mixture with ethyl acetate, dried in a vacuum dessicator, and resuspended in 30 μl of ethyl acetate. Samples were applied to thin-layer chromatography (TLC) plates and the acetylated and non-acetylated forms of chloramphenicol were separated for 30 minutes at room temperature with chloroform/methanol (95/5% v/v) as the mobile phase. Signals were

then visualized and quantified by electronic autoradiography (Instantimager, Packard). To assess β-gal activity, 20 μl of the muscle extracts were combined with 130 μl of water and an equal volume (150 μl) of assay buffer, consisting of 120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol and 1.18 mM 0-nitrophenyl-β-D-galactopyranoside. The reaction mixture was then incubated at 37°C for 2.5 hours, and β-gal activity was determined photometrically at 420 nm. Reactions containing extracts derived from muscles which had not been transfected with the β-gal construct possessed an endogenous enzyme activity (7) ranging from 0.045-0.069 absorbance units/hr. There also appeared to be a modest effect of stimulation on endogenous β-gal activity, since this activity was elevated by 25-30% from day 1 to day 7 (results not shown). Thus, in order to use β-gal expression to correct CAT activity for transfection efficiency, we calculated the actual transfected β-gal activity by subtracting the endogenous activity from the total activity measured during the time course of the experiment.

In vitro mRNA decay. Protein extracts from stimulated and non-stimulated control EDL muscles were prepared according to previously published protocols (15, 36), with some modifications. Briefly, skeletal muscle powders (50-100 mg) were homogenised (Ultra Turrax, 7 mm probe) 3 x 10 sec (70% maximum) in 1 ml of homogenisation buffer comprised of 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM HEPES, 0.5 mM DTT and 0.5 mM PMSF. These homogenates were then centrifuged for 15 min at 5,000g (Beckman, Avanti J-25) at 4°C. The resulting supernatant fractions were then subjected to further centrifugation at 15,000g (4°C) for 15 minutes and the resultant post-

mitochondrial supernate (S15) was transferred to a sterile eppendorf tube. Protein concentrations of the S15 fractions from stimulated and control muscles were determined photometrically (2). Total RNA (30 or 60 μg) from stimulated or control EDL muscles was incubated with 60 or 200 μg of S15 extract obtained from either stimulated or control muscles in a 300 μl reaction volume at 37°C. Aliquots were removed at various times as indicated in the Figure Legends, and the RNA was extracted using a phenol/chloroform/isoamyl alcohol extraction procedure. The RNA was then precipitated at -70°C for 1 hour, and subsequently pelleted, dried and resuspended in 10 μl of sterile H_2O. The RNA was separated on a 1% agarose gel, transferred to a nylon membrane (Hybond-N, Amersham), and fixed to the membrane with ultraviolet light. These membranes were probed with ^{32}P-dCTP labelled cDNAs specific for cytochrome c mRNA, and signals were quantified using electronic autoradiography (Instantimager, Packard).

Statistical analyses. The effects of chronic stimulation on muscle mass, total protein, total RNA, cytochrome c mRNA and cytochrome c protein levels were evaluated using paired t-tests. Changes in cytochrome c transcriptional activation and mRNA stability following increased contractile activity were determined using two-way analyses of variance, followed by Tukey's post-hoc test. All values are presented as mean ± S.E.M. and differences were considered to be statistically significant at the 0.05 level of confidence.

RESULTS

Body mass, TA muscle weights, total protein and total RNA concentrations. Chronic contractile activity had no effect on body mass or TA muscle mass over the 7 day time period (Table 1). Control TA muscle mass averaged 485.9 ± 12.8 mg (n = 43). Total protein concentration in control muscle averaged 84.7 ± 4.4 mg/g wet weight (n = 27), and was not influenced by contractile activity. The total RNA concentration in non-stimulated control TA muscle was 1846.2 ± 77.2 μg/g (n = 34) and was not significantly affected by the simulation protocol (Table 1).

Cytochrome c mRNA and protein levels. Chronic contractile activity induced an increase in steady-state cytochrome c mRNA content in the TA muscle, from 1.3-fold on day 1, to 1.7-fold between days 5 and 7 ($p<0.05$; Fig 1A, 1C). The 1.5-fold increases apparent at days 2 and 4 tended toward, but did not attain statistical significance ($0.05<p<0.1$). There was no effect of contractile activity on 18S rRNA levels, as reported previously (28). The time course of the stimulation-induced alterations in cytochrome c mRNA was different from that observed for cytochrome c protein content (Fig. 1C). No changes in protein level were evident until 5 days of stimulation (Figs. 1B, 1C). Cytochrome c protein remained elevated in the stimulated TA muscle at 7 days of stimulation, attaining levels which were 1.9-fold above those observed in control muscle (Fig. 1C). These increases in expression are typical of those observed using this chronic stimulation model (14, 27-29, 36).

Transfected β-galactosidase activity in control and stimulated TA muscles. Transfected β-gal activity, used as a correction factor for transfection efficiency, varied

between 0.05-0.08 absorbance units/hr over the 7 day experimental period. There was no effect of time or stimulation on transfected β-gal activity.

Cytochrome c transcriptional activation. Initial studies were performed using control, unstimulated TA muscles to confirm that transcriptional activation of the cytochrome c promoter would occur following injection with the cytochrome c DNA constructs. Transcriptional activation of the pRC4CAT/-66 construct was only marginally above background levels of detectability (n=8). In contrast, CAT activity driven by the pRC4CAT/-326 construct was much higher than background levels, and it was found to progressively increase over time following injection, even as much as 5 weeks later (results not shown). These initial studies confirmed the effectiveness of our injection protocol, verified the stability of the transcriptional activation product (CAT) in our injected muscles, and led us to the use of the pRC4CAT/-326 construct in evaluating the effect of contractile activity on cytochrome c transcriptional activation.

A time-dependent increase in cytochrome c promoter-driven CAT activity was evident over the 7 day period in non-stimulated TA muscle. An approximate 4-fold accumulation of corrected CAT activity above that found at day 1 was evident between days 4 and 7 of the experimental protocol (Fig. 2B; n=27). In the stimulated muscle, an increase in CAT activity was only evident after 5 days of contractile activity, reaching values that were 2.1-fold higher ($p<0.05$) than those measured in contralateral, non-stimulated TA muscles (Figs. 2A, 2B, n=11).

Cytochrome c mRNA stability. In establishing the *in vitro* mRNA decay assay

conditions, we first showed that mRNA stability was unaffected in the presence of the individual components of the decay reaction. We also documented that mRNA levels progressively decreased as a function of incubation time (5, 10, 20, and 40 min) in the presence of cytosol, and that decay was linear with the amount of cytosolic fraction added (20, 40 and 66 μg protein/lane; Connor and Hood, unpublished observations). The assay conditions chosen (10 and 20 min; 20 μg protein/lane) represent the outcome of those preliminary studies. We assessed the effect of contractile activity on cytochrome c mRNA stability at each time point of the 7 day experimental protocol. Since similar results were observed in 2, 3 and 4 day stimulated and non-stimulated muscle, only the data obtained from muscle subjected to 3 days of contractile activity are illustrated (Fig. 3*A*). When total RNA was incubated with cytosol isolated from the same muscle, cytochrome c mRNA derived from the stimulated muscle (Fig. 3*A* lanes 1-3) was degraded at a slower rate than cytochrome c mRNA from control muscle [Fig. 3, *A* (lanes 3-6) and *B*]. This contractile activity-mediated increase in cytochrome c mRNA stability was no longer evident following 5 days of stimulation, when rates of degradation in the stimulated muscle were equivalent to the cytosol-induced degradation of cytochrome c mRNA from control muscle [Fig. 4, *A* (lanes 1-3 vs. lanes 4-6) and *B*]. These results at 5 days were also similar to rates of cytochrome c mRNA decay at both 1 and 7 days (not shown).

In a separate subset of animals, we further evaluated the role of the cytosolic fraction in mediating cytochrome c mRNA stability under conditions of continuous contractile activity (24 hr/day for 10 days), similar to that used recently (36). This treatment resulted

in a 1.9-fold increase in cytochrome c mRNA in the stimulated muscle. In these decay reactions, cytosolic fractions obtained from both stimulated and control muscles were incubated only with RNA isolated from control muscle. Cytochrome c mRNA was more stable in the presence of cytosolic fraction derived from the muscle subject to continuous chronic contractile activity (Fig. 5). The $t_{½}$ value (26 min) was 88% higher in the presence of the cytosol from stimulated muscle, compared to the value obtained in the presence of the control cytosol (14 min). These data are consistent with the concept hypothesized by Yan et al. (1996) that a reduced expression of a destabilizing factor occurs in response to chronic contractile activity. Consequently, there is a reduction in the degradation rate of cytochrome c mRNA.

DISCUSSION

Contractile activity is a potent stimulus for the induction of mitochondrial biogenesis in skeletal muscle (cf. 10, 12 for reviews), and thus serves as a good experimental model to study the underlying mechanisms involved in organelle synthesis and turnover. In the present study, contractile activity was induced using a chronic low-frequency electrical stimulation protocol (10 Hz, 3 hrs/day), which has been shown to lead to mitochondrial phenotypic alterations, as well as increases in muscle mitochondrial content (28, 29). In order to document some of the underlying mechanisms involved in these adaptations, we chose to measure cytochrome c protein and mRNA levels, as well as the attendent processes of gene transcription and mRNA stability. The results indicate that increases in cytochrome c expression brought about by chronic contractile activity are due to time-dependent adaptations, which appear to involve an increase in mRNA stability, followed by a subsequent increase in transcriptional activation.

The application of a cell-free mRNA decay assay (21) to measure mRNA degradation in the presence of a cytosolic fraction permitted the conclusion that the early increases in cytochrome c mRNA expression were due to a change in mRNA stability. The mRNA decay assay employed appears to be sensitive enough to measure subtle alterations in mRNA stability, even when studying mRNAs possessing relatively low rates of turnover, as found in skeletal muscle (6). The cellular events mediating the stabilization of cytochrome c mRNA as a result of contractile activity likely involve the 3′ untranslated region (UTR), since it is established that this is an important site of RNA-protein interactions which confers

alterations in mRNA stability (22, 23). Yan et al. (36) recently demonstrated a reduced RNA-protein interaction in the 3′ UTR of cytochrome c mRNA in response to 9 days of stimulation (24 hrs/day), at a time when cytochrome c mRNA levels had increased over 2-fold. This suggested that contractile activity induced the activation of an inhibitor of RNA-protein interactions. This important observation is probably related to our findings, since we provide direct evidence for an increase in cytochrome c mRNA stability brought about by a factor endogenous to the cytosol of 10 day, continuously stimulated muscle. We also show that an increase in stability can be a relatively early occurrence as a result of contractile activity, since the effect appeared after only 2 days of treatment when the muscle was allowed a recovery phase. As with the induction of the transcription factors responsible for transcriptional activation (see below), we hypothesize that the recovery phase may have permitted the induction of an mRNA stabilizing factor, however its characterization and its effect on other nuclear-encoded mRNAs remain to be determined. It is interesting to note that the inhibition of mitochondrial protein synthesis in Hep G2 hepatocytes leads to an increase in the stability of a variety of nuclear-encoded mRNAs (5). In this context, we have recently observed that 5 minutes of 10 Hz contractile activity markedly reduced mitochondrial protein synthesis in subsarcolemmal mitochondria (M.K. Connor, O. Bezborodova and D.A. Hood, unpublished observations). This decrease in intra-mitochondrial translation rate may be transmitted to the nuclear genome via a putative signal (18) leading to the induction of stabilizing proteins (5).

The early mRNA stabilization was followed by an increase in transcriptional

activation, leading to an eventual accumulation of cytochrome c mRNA, evident prior to an increase in protein level (Fig. 1). Our assessment of transcriptional activation was afforded by the availability of the pRC4CAT/-326 bp cytochrome c promoter/reporter DNA construct (11). The technique of direct plasmid DNA injection into muscle, one which is useful for the investigation of transcriptional events in skeletal muscle during alterations in contractile activity (4, 26), was employed. This technique requires the co-injection of a non-specific reporter gene to correct for transfection efficiency. In the present study, muscles were injected with plasmid DNA containing a RSV promoter linked to a β-galactosidase reporter gene. An endogenous muscle β-galactosidase activity consistent with reports in the literature (7) was measured, and we also found that total (endogenous + transfected) β-galactosidase enzyme activity was progressively elevated in response to 7 days of muscle stimulation (results not shown). There was no effect of contractile activity on transfected β-galactosidase activity, and this value was used to correct CAT activity for transfection efficiency. It should be noted however, that a variety of stressors have been reported to activate the CMV promoter in other systems (3). Thus, the use of RSV or CMV promoter-driven reporter constructs must be used with caution in skeletal muscle subject to chronic contractile activity.

Recently, the transcriptional activation of cytochrome c in cardiac myocytes subject to electrical pacing in cell culture was reported (34). Both the NRF-1 and cAMP response element sites are important responsive elements within the cytochrome c promoter which mediate transcription under those conditions. The cAMP response element site appears to

bind c-Jun, and both NRF-1 and c-jun mRNA levels increase in response to cardiac pacing (35). In skeletal muscle, it is known that c-jun mRNA is increased as a result of contractile activity (16). It is noteworthy that the pattern of induction of immediate early genes such as c-*fos*, c-*jun* and *egr-1* is particularly pronounced during the 0.5-8 hour recovery phase after the cessation of contractile activity (1, 17, 19). This is well within the time frame of the 21 hour recovery period which we employed between each 3 hour bout of contractile activity. As a result, with repeated 3 hour bouts of contractile activity followed by recovery, a progressive accumulation of transcription factors (eg. c-Jun and NRF-1) could have occurred, leading to the transcriptional activation which we observed after five days of contractile activity. Thus, the recovery period may have allowed for an earlier onset, but not necessarily a greater magnitude, of cytochrome c mRNA induction in comparison to continuous contractile activity (36) using the same absolute workload (i.e. 10 Hz).

In summary, we used the technique of direct plasmid DNA injection to measure transcriptional activation, combined with a sensitive mRNA decay assay to measure mRNA stability. The increase in cytochrome c mRNA in skeletal muscle undergoing contractile activity-induced mitochondrial biogenesis can be explained largely by the existence of time-dependent, rapid increases in mRNA stability, followed by increases in transcriptional activation. These changes precede increases in cytochrome c protein expression. The data suggest the utility of these two complimentary experimental approaches for the study of muscle adaptations, and identify mRNA stability as a rapidly altered physiological process which deserves greater attention in the area of skeletal muscle gene expression.

Footnote:

[1]Michael Connor and Damien Freyssenet contributed equally to this study.

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Table 1. *Body mass, tibialis anterior muscle mass, total protein and total RNA concentrations during chronic stimulation.*

	Days of stimulation					
Parameter	1	2	3	4	5	7
Body mass (g)	255 ± 17	277 ± 21	270 ± 7	261 ± 8	283 ± 9	303 ± 11
Muscle mass (S/C)	1.01 ± 0.03	1.06 ± 0.03	0.99 ± 0.01	1.07 ± 0.02	1.01 ± 0.01	1.02 ± 0.02
Total protein (S/C)	0.92 ± 0.05	0.89 ± 0.07	0.99 ± 0.08	0.95 ± 0.04	1.03 ± 0.10	0.97 ± 0.06
Total RNA (S/C)	1.35 ± 0.18	1.28 ± 0.15	1.46 ± 0.25	1.35 ± 0.23	1.11 ± 0.07	1.21 ± 0.07

Values are mean ± S.E.M.; n = 4-10 rats/group; tibialis anterior muscle mass, total protein and total RNA concentrations are expressed as ratios of levels obtained in stimulated (S) muscles to those obtained in non-stimulated control (C) muscles.

Fig. 1. Representative northern (A) and western (B) blots measuring the steady-state cytochrome c mRNA and protein levels, respectively, in 3 hour/day stimulated (S) and non-stimulated control (C) muscle. C) Graphical representation of multiple northern and western blots illustrating the time course of stimulation-induced changes in muscle cytochrome c mRNA (■) and protein (□) levels ($n = 3$, 1 d; $n = 3$, 2 d; $n = 4$, 3 d; $n = 4$, 4 d; $n = 11$, 5 d; $n = 9$, 7 d). * $p<0.05$, stimulated vs. contralateral, non-stimulated control cytochrome c mRNA; ¶, $p<0.05$, stimulated vs. contralateral, non-stimulated control cytochrome c protein.

Fig.2. Cytochrome c transcriptional activation, measured as chloramphenicol acetyltransferase (CAT) activity in stimulated (S) and non-stimulated control (C) skeletal muscle injected with 50 μg of a plasmid DNA construct comprised of the -326 bp cytochrome c promoter linked to a CAT reporter gene. A) Representative autoradiogram of CAT activity. B) Time course response of CAT activity from multiple sets of animals ($n = 3$, 1 d; $n = 4$, 2 d; $n = 3$, 3 d; $n = 3$, 4 d; $n = 12$, 5 d; $n = 7$, 7 d; *$p<0.05$, stimulated vs. contralateral, non-stimulated control).

Fig. 3. A) Autoradiogram of an *in vitro* cytochrome c mRNA decay reaction after 3 days of contractile activity (3 hours/day). Total RNA (10 μg/lane) isolated from stimulated muscle (S) was incubated either in the presence of buffer (lane 1 at t = 0) cytosol (20 μg/lane) obtained from the same stimulated muscle (lanes 2-3) for 10 or 20 minutes. Similar incubations were conducted using RNA isolated from non-stimulated control muscle (C; lanes 4-6). B) Graphical representation of multiple cytochrome c mRNA decay experiments comparing 3 day stimulated (■) and non-stimulated control (▲) skeletal muscle (n = 5 animals; * $p<0.05$, stimulated vs. contralateral, non-stimulated control).

Fig. 4. A) Autoradiogram of an *in vitro* cytochrome c mRNA decay reaction after 5 days of contractile activity (3 hours/day). Total RNA (10 μg/lane) isolated from stimulated muscle (S) was incubated either in the presence of buffer (lane 1 at t = 0) cytosol (20 μg/lane) obtained from the same stimulated muscle (lanes 2-3) for 10 or 20 minutes. Similar incubations were conducted using RNA isolated from non-stimulated control muscle (C; lanes 4-6). B) Graphical representation of multiple cytochrome c mRNA decay experiments comparing 3 day stimulated (■) and non-stimulated control (▲) skeletal muscle (n = 3 animals).

Fig. 5. A) Autoradiogram of an *in vitro* cytochrome c mRNA decay reaction after 10 days of contractile activity (24 hours/day). Total RNA (12 μg/lane) from non-stimulated control (CTL) skeletal muscle was incubated either with cytosol (40 μg/lane) from stimulated muscle (STIM) or with cytosol from control muscle for 5, 10, 20 or 40 minutes. B) Graphical representation of multiple cytochrome c mRNA decay experiments comparing 10 day stimulated (■) and non-stimulated control (▲) skeletal muscle (n = 6 animals; * $p<0.05$, stimulated vs. contralateral, non-stimulated control).

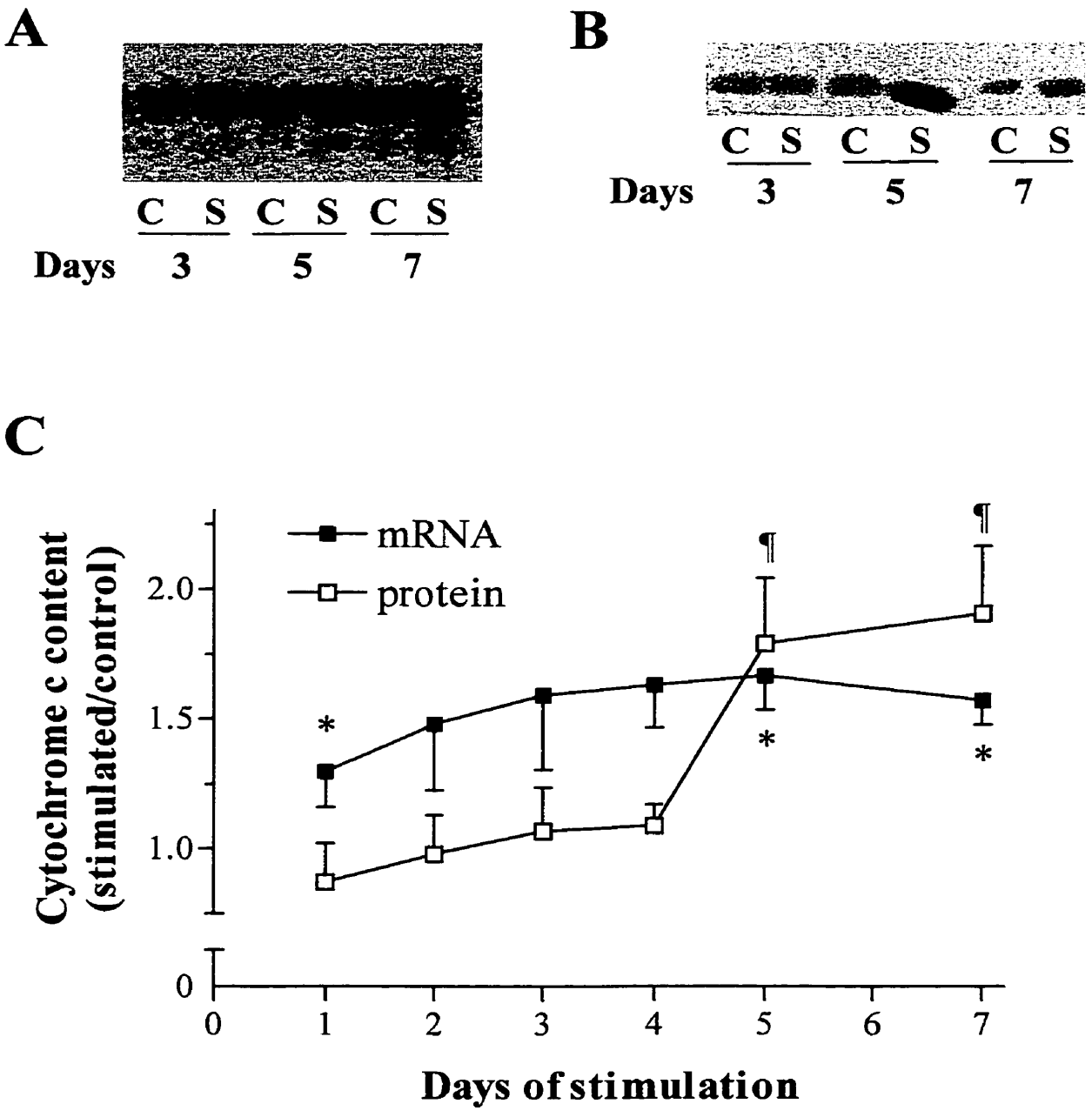
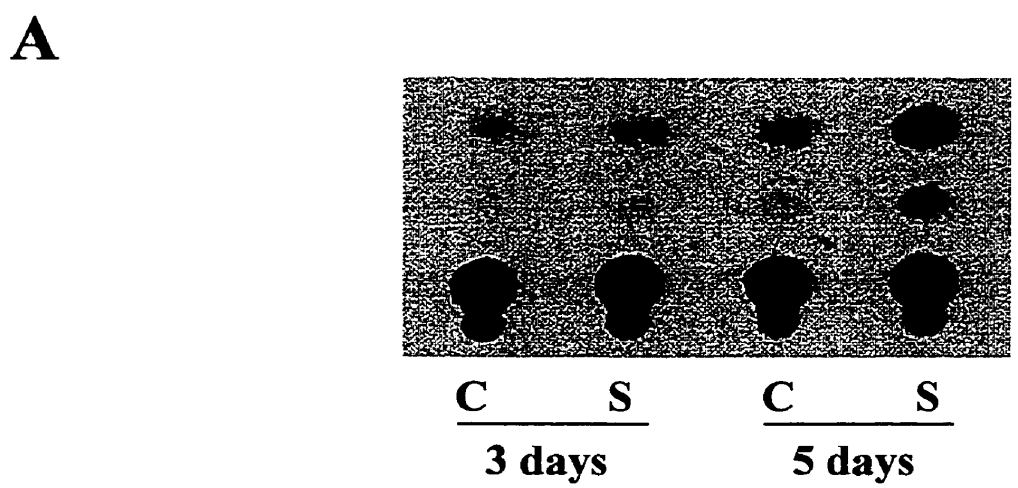

A
C S C S C S
Days 3 5 7
B
C S C S C S
Days 3 5 7
C
mRNA
protein
Cytochrome c content (stimulated/control)
2.0
1.5
1.0
0
0 1 2 3 4 5 6 7
Days of stimulation
*
*
*
¶
¶

Figure 1


A
C
S
C
S
3 days
5 days

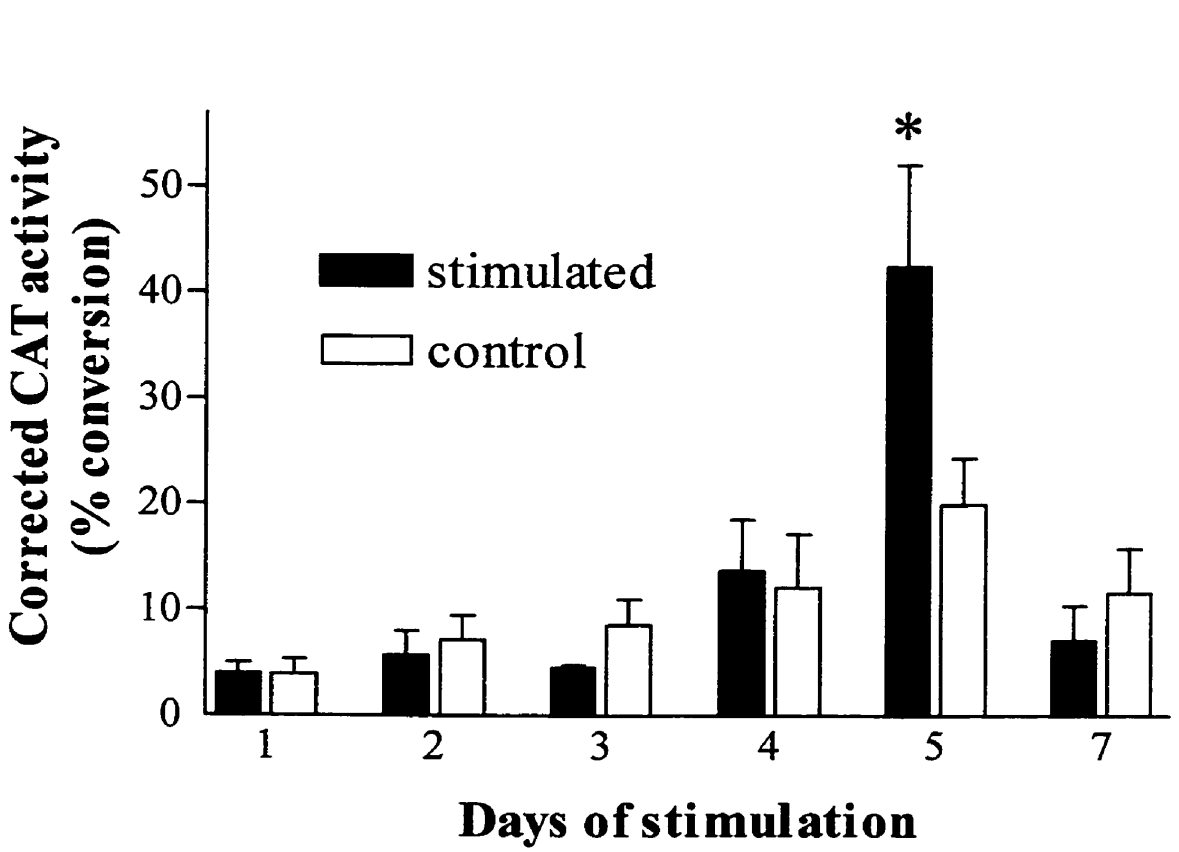

B
Corrected CAT activity
(% conversion)
50
40
30
20
10
0
stimulated
control
*
1
2
3
4
5
7
Days of stimulation

Figure 2

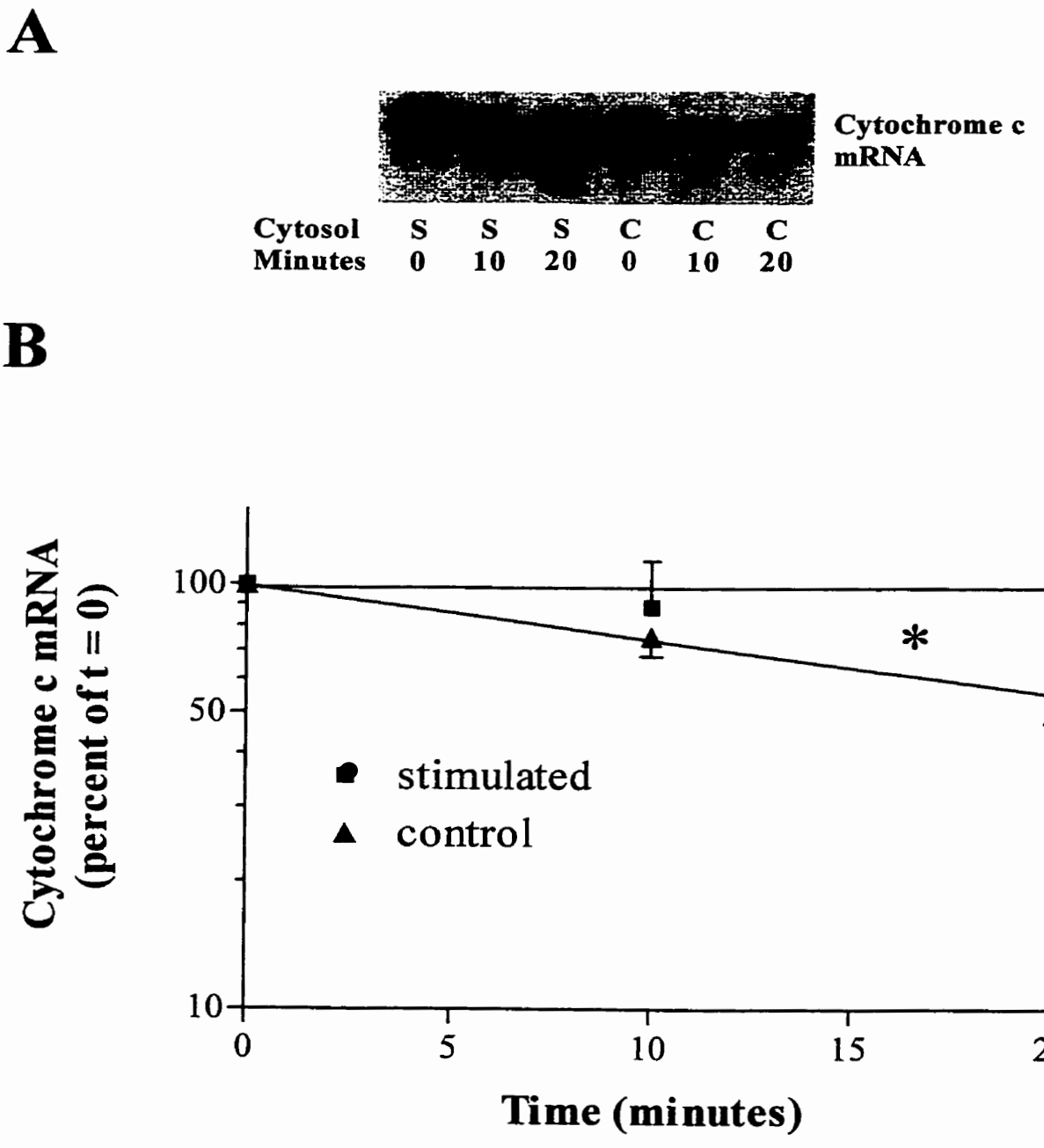

A
Cytochrome c mRNA
Cytosol S S S C C C
Minutes 0 10 20 0 10 20
B
Cytochrome c mRNA (percent of t = 0)
100
50
10
stimulated
control
*
0
5
10
15
20
Time (minutes)

Figure 3

A

Cytochrome c mRNA

Cytosol	S	S	S	C	C	C
Minutes	0	10	20	0	10	20

B

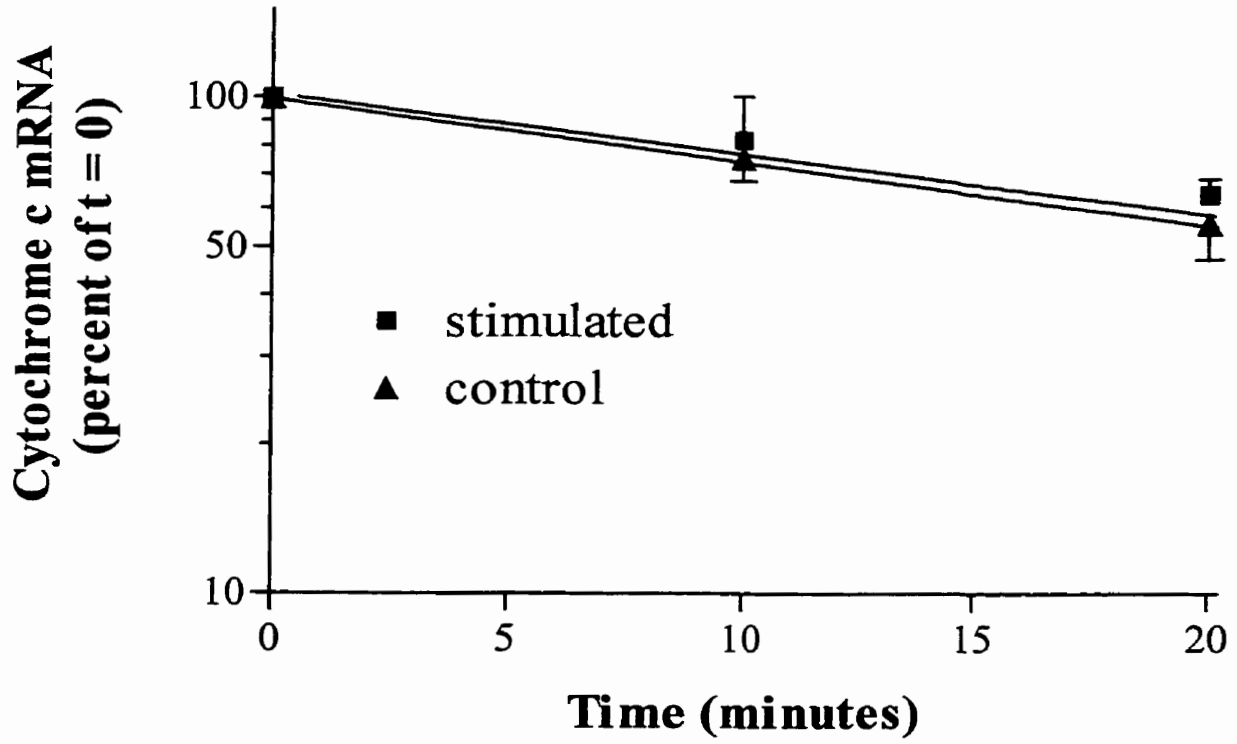


Figure 4

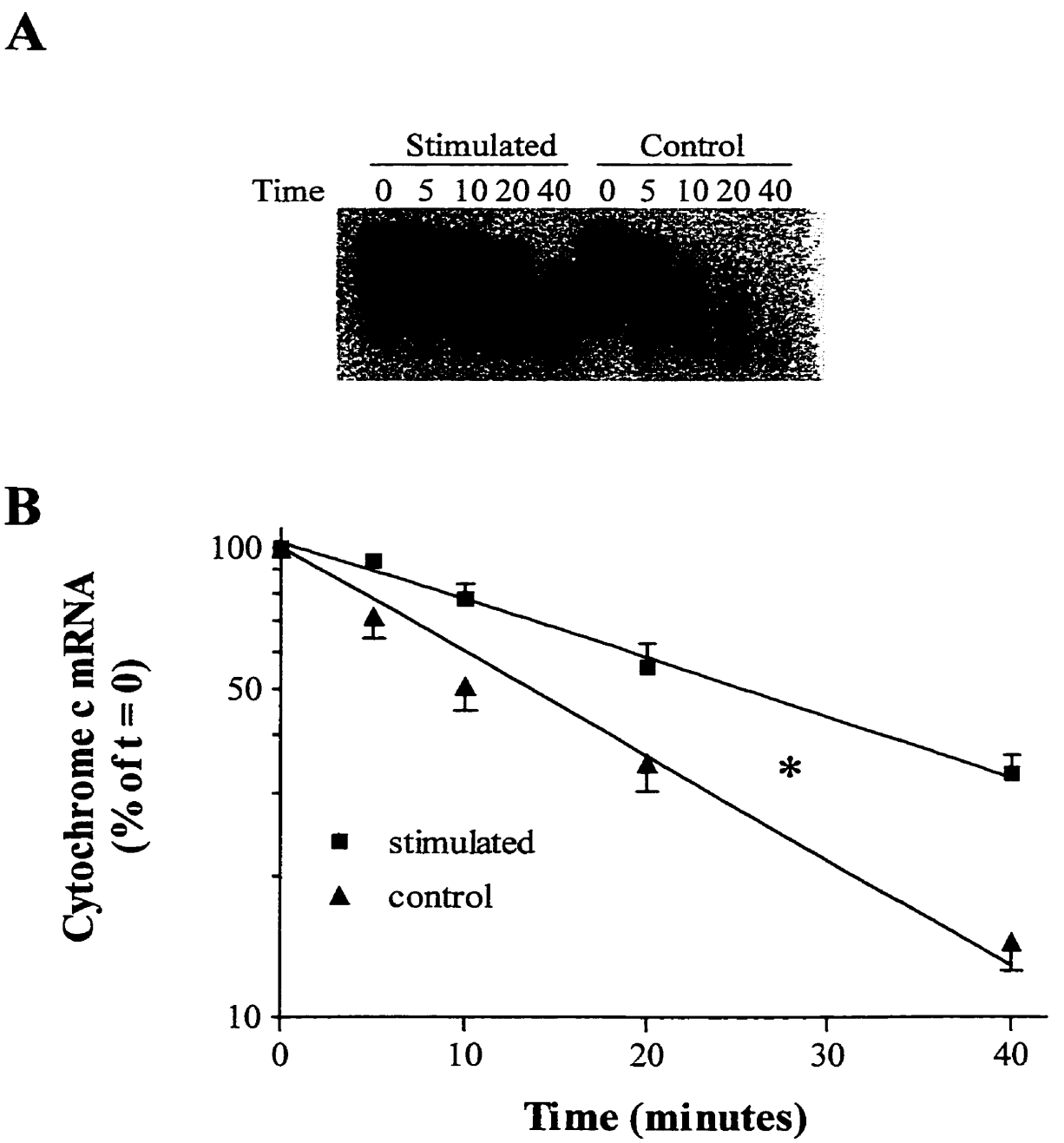

A
Stimulated
Control
Time
0 5 10 20 40 0 5 10 20 40
B
Cytochrome c mRNA
(% of t = 0)
100
50
10
0
10
20
30
40
Time (minutes)
stimulated
control
*

Figure 5

Rationale for Manuscript # 3

The response of skeletal muscle to contractile activity is very complex and the mechanisms responsible for these adaptations remain relatively poorly understood. This is due to the fact that most studies use an *in vivo* whole animal model to evaluate activity-induced gene expression. Although this model remains extremely important in terms of the *in vivo* response of skeletal muscle to contractile activity, it does not allow for the use of some precise manipulations which are routinely used in molecular biology, making the definition of the cellular mechanisms responsible for these changes difficult. This study was designed to develop a contracting skeletal muscle cell culture model in order to define the response of muscle to contractile activity at the molecular level. This will allow for the manipulation of the expression of specific proteins within the cell as well as the dissection of the events of muscle contraction which may initiate activity-induced adaptations. Thus, this model may lead to a better understanding of the potential mechanisms that underlie the responses of skeletal muscle to contractile activity *in vivo*. These data may provide the groundwork for the definition of precise mechanisms that regulate skeletal muscle gene expression, which can lead to a better understanding of the maladies that affect skeletal muscle.

I was responsible for all of the experimental work described in this paper and Dr. Hood served as the principal investigator of the study.

MANUSCRIPT # 3:

Contractile Activity-Induced Transcriptional Activation of Cytochrome c involves Sp1, and requires cross-bridge cycling in C2C12 Muscle Cells.

To be submitted to J. Biol. Chem.

Contractile Activity-induced Transcriptional Activation of Cytochrome c involves Sp1, and requires cross-bridge cycling in C2C12 Muscle Cells*

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[1]The abbreviations used are: ACT, actinomycin D; ATPase, adenosine triphosphatase, BAPTA-AM, 1,2-bis(*o*-Aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester; BDM, 2,3-butanedione monoxime; $[Ca2+]_{ic}$, intracellular Ca^{2+} concentration; CAT, chloramphenicol acetyltransferase; DEPC, diethyl pyrocarbonate; DMSO, dimethyl sulfoxide; TTX, tetrodotoxin;

[2]M.K. Connor and D.A. Hood, unpublished observations.

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SUMMARY

Contractile activity is known to induce adaptations in the expression of genes encoding skeletal muscle mitochondrial proteins, however the putative signals responsible for these adaptations remain unknown. We used electrical stimulation (5 Hz, 65 V) of C2C12 muscle cells in culture to define the underlying mechanisms which underlie contractile activity-induced adaptations in the expression of nuclear-encoded mitochondrial genes. Acute contractile activity elevated egr-1 mRNA levels 4.0-fold above those in non-stimulated cells after only 30 min of stimulation. Chronic contractile activity (4 days, 3 h/day) augmented cytochrome c mRNA to levels which were 1.6-fold higher than in control cells. Overexpression of the wild-type (wt) transcription factor Egr-1 increased cytochrome c mRNA levels 2.2-fold above those in non-stimulated cells, and the combination of Egr-1 and electrical stimulation elevated cytochrome c mRNA 3.6-fold above control, likely mediated by a combination of increased mRNA stability and transcriptional activation. Overexpression of a mutant form of Egr-1 abolished this response. Cells transfected with full-length cytochrome c promoter/CAT reporter constructs demonstrated that the contractile activity-induced increases in cytochrome c mRNA were a result of transcriptional activation of the cytochrome c gene. This response was no longer evident following pre-treatment of cells with either TTX, BAPTA-AM or BDM, which inhibited muscle contraction at various levels. A similar response was apparent when the minimal cytochrome c promoter was used, suggesting that the factors responsible for this transcriptional activation bind within this -66 to +115 region. DNA binding assays revealed an elevated binding within the first intron (+75

to +104 bp) in response to increased contractile activity, which was paralleled by augmentations in Sp1, but not Egr-1, DNA binding. These data suggest that cross-bridge cycling and the attendant ATP utilization are necessary events for cytochrome c transactivation, and that Sp1 responds to these events by mediating an increase in cytochrome c gene transcription.

Elevations in skeletal muscle contractile activity are known to induce large and rapid augmentations in the mRNA expression of genes encoding mitochondrial proteins (1,2,3). These increases result from a disruption in the equilibrium that exists between gene transcription and mRNA stability during non-adaptive steady-state conditions. Elevations in the level of the mRNA encoding the nuclear gene product cytochrome c in response to *in vivo* contractile activity have been reported (4), and our recent work has established that this adaptation is mediated via sequential, time-dependent elevations in both of these processes (5). This necessitates alterations in the expression of proteins involved in regulating transcriptional activation, and/or message stabilization within the cytosol. However, the specific cellular events which occur during muscle contraction to initiate these adaptations remain largely undefined. These events could include membrane depolarization, Ca^{2+} mobilization, cross-bridge cycling and alterations in energy metabolism. Each of these are known to initiate intracellular signaling cascades (6-10) which could ultimately alter rates of transcription and/or mRNA stability. It is now established that acute elevations in contractile activity can stimulate a number of kinases involved in signal transduction, including mitogen activated protein kinase (MAP kinase), c-jun N-terminal kinase and p38 kinase activities in skeletal muscle (11, 12). However, the temporal relationship between the onset of the putative signalling event (i.e. kinase activity), transcription factor activation and the up-regulation of nuclear genes encoding mitochondrial proteins in response to contractile activity, remains unknown.

We have previously used cytochrome c as a model, nuclear-encoded mitochondrial

protein, to define some of the adaptations which occur in response to contractile activity and artificially elevated muscle Ca^{2+} levels (5, 7). The cytochrome c promoter contains multiple GC-rich regions within its promoter, which can serve as binding sites for the transcription factor Sp1 (13-15). However, the zinc finger transcription factor Egr-1 (16) may also interact with these elements, since the consensus binding sequences are similar, and it may be that Sp1 and Egr-1 can compete for similar, non-consensus sites (13). Furthermore, Sp1-mediated gene transcription is inhibited by Egr-1 displacement of Sp1 from the promoter (17, 18). In addition, Egr-1 is rapidly induced in response to elevations in contractile activity (19, 20) as well as elevated intracellular Ca^{2+} levels (21). These findings suggest potential roles for both Sp1 and Egr-1 in the regulation of cytochrome c expression during conditions of increased contractile activity.

Thus, in order to define some of the events occurring during muscle contraction which initiate the increase in cytochrome c expression observed, and to identify some of the transcription factors which are immediately responsible for this increase, we adopted a contracting C2C12 murine skeletal muscle cell model. Our results indicate that 1) cytochrome c mRNA levels are increased by contractile activity, 2) these adaptations result from increases in gene transcription and 3) transcriptional activation appears involve elevations in Sp1 binding within the first intron. In addition, cross-bridge cycling is necessary to confer these activity-induced adaptations in cytochrome c gene expression.

EXPERIMENTAL PROCEDURES

Materials - BDM, TTX and ACT were purchased from Sigma (St. Louis, MO). [^{14}C]chloramphenicol, [$^{\alpha}$32P]dCTP, nitrocellulose and nylon membranes (Hybond N) were obtained from Amersham-Pharmacia Biotech (Baie d' Urfé, PQ, Canada). BAPTA-AM was purchased from Calbiochem (San Diego, CA). Fetal bovine serum and horse serum were obtained from Summit Biotechnologies (Fort Collins, CO) and Life Technologies (Burlington, ON, Canada), respectively. Polyclonal antibodies directed towards Egr-1, Sp1 and Cyclin D1 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All other reagents were purchased from Sigma and were of the highest grade available. The oligodeoxynucleotides used for electromobility shift assays were a) Sp1, 5'-ATTCGATCGGGGCGGGGCGAGC-3' (Dalton Chemicals, Toronto); b) Egr-1, 5'-GGATCCAGCGGGGGCGAGCGGGGGCGA-3' (Santa Cruz Biotechnology, Santa Cruz); and c) a sequence comprised of +75 to +104 of the first intron of the cytochrome c gene, 5'-GGGGACGCGGGGCGGGAAGAGGGCGAGGAG-3' (Dalton Chemicals, Toronto).

Cell Culture - C2C12 murine skeletal muscle cells were maintained at 37°C in 5% CO_2 on 100 mm gelatin-coated plastic dishes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (P/S). Upon reaching 90% confluence, myoblast differentiation was induced by switching to a lower serum medium (DMEM supplemented with 5% heat-inactivated horse serum and 1% P/S). Treatments were routinely carried out when the myotubes reached 90-100% confluence

(approximately 8-10 days).

Electrical Stimulation - The design of the electrical stimulation apparatus was derived through the modification of existing methods (22-24). Lids from plastic 100 mm culture dishes (Sarstedt, Montreal, PQ, Canada) were fitted with two platinum wire electrodes such that 5 cm lengths ran parallel to each other at opposite ends of the dish approximately 5 cm apart. Electrodes were attached to the plates using banana plugs (Electrosonic, North York, ON, Canada). Multiple plates were arranged in parallel and connected to a stimulator unit which reversed the polarity of the output every second. Cells were stimulated at a frequency of 5 Hz and an intensity of 65 V ($1.2V/cm^2$). Stimulation was either performed acutely for 5, 15, 30, 60 or 240 min or chronically for 4 days (3 h/day).

Inhibition of Muscle Contraction - To determine the event(s) of muscle contraction responsible for activity-induced alterations in gene expression, sarcomere shortening was inhibited at three different levels 24 h prior to the onset of stimulation: 1) Membrane depolarization was inhibited by preventing the opening of voltage-gated Na^+ channels by treatment with 10 μM TTX; 2) Contraction was disrupted by pre-treatment with the membrane-permeable Ca^{2+} chelator BAPTA-AM (25 μM). This was done to prevent the increase in cytosolic [Ca^{2+}] which occurs subsequent to membrane depolarization; 3) Contraction was inhibited by preventing cross-bridge cycling with 1.5 mM BDM. This concentration of BDM has been shown to have no effect on Ca^{2+} transients in phenylepherine treated cardiac myocytes (25) and likely acts to inhibit myosin ATPase activity (26). Cells were electrically stimulated for 4 days during each treatment (3 h/day) and results from drug-

treated cells were compared to quiescent cells treated with the corresponding vehicle (VEH).

Steady-state mRNA Measurements - Total RNA was isolated from stimulated and non-stimulated control cells as done previously (7) and resuspended in DEPC-treated H_2O. Determination of the quality and subsequent size separation of total RNA (30 μg) was achieved by electrophoresis using denaturing formaldehyde-1% agarose gels, which were transferred and subsequently fixed to nylon membranes. These membranes were then probed with radiolabelled cDNA probes encoding cytochrome c, egr-1 and 18S rRNA as done previously (27). Stringent washes were performed at 55°C for 15 min in 0.1 x SSC/0.1 % SDS followed by 15 min at 60°C. Signals were quantified by electronic autoradiography (Instantimager, Packard) and cytochrome c and egr-1 mRNAs were normalized to 18S rRNA levels to correct for uneven loading.

Plasmids - Plasmid constructs containing various lengths of the cytochrome c promoters linked to a reporter gene (pRC4CAT) were provided by Dr. Richard Scarpulla (Northwestern University, Chicago, IL) and have been previously characterized (15, 28). The pRC4CAT/-726 construct is comprised of sequences from the cytochrome c promoter from -726 bp upstream of the transcription start site to position +115 within the first intron, fused to a chloramphenicol acetyltransferase (CAT) reporter gene. Experiments utilizing the minimal cytochrome c promoter (pRC4CAT/-66) were also conducted, as this region has been shown to be sufficient to confer cytochrome c transcriptional activation in response to elevated levels of intracellular Ca^{2+} (7). β-galactosidase activity was under the control of pRSV/β-gal. Overexpression of wild-type (wt; pCMV/930) or mutant (mt; pCMV/858) Egr-1 was

achieved with vectors provided by Dr. Vikas Sukhatme (Harvard University, Boston, MA). The mt Egr-1 lacks amino acids 331-374 in its DNA-binding domain and is DNA-binding deficient.

DNA Transfection and Expression Assays - C2C12 myoblasts were transfected with the appropriate cytochrome c promoter/CAT construct (5 μg/100 mm dish) when they reached 70% confluence. Cells were co-transfected with pRSV/β-gal (5 μg/dish) to correct for differences in transfection efficiency. Where applicable, wt or mt expression vectors were transfected (5 μg/dish) in combination with cytochrome c promoter/CAT constructs. The total amount of DNA added was maintained constant in all transfected cells. Transfections were done using a poly-L-ornithine method followed by a DMSO shock (29) and cells were then differentiated by switching to a low serum medium. A subgroup of cells were treated with 10 mM TTX, 25 mM BAPTA-AM or 1.5 mM BDM 24 h prior to the onset of stimulation where indicated. CAT and β-galactosidase activities in stimulated and quiescent cells were measured as described previously (7).

Measurement of mRNA Stability - Myoblasts were transfected with 5 μg of ev or wt Egr-1 plasmid DNA when they reached 80% confluence. Following 8-11 days of differentiation, transfected myocytes were either electrically stimulated for 4 days (3 h/day) or left untreated for a similar time period. Immediately after the final bout of stimulation, cells (stimulated and control) were treated with either 10 μg/ml actinomycin D (ACT) to inhibit mRNA synthesis or an equivalent volume of methanol which served as the vehicle (VEH). Myocytes were allowed to incubate with ACT or VEH for 4, 24 or 48 hours. At the

appropriate time points cells were harvested and total RNA was isolated. Equal amounts of total RNA (15 μg) from ACT- and VEH-treated cells were subjected to Northern blotting as described above. Blots were probed for cytochrome c mRNA and uneven loading was corrected using 18S rRNA. Cytochrome c mRNA degradation was assessed by expressing the mRNA levels found at all time points as a percent of the t=0 value.

Electromobility Shift Assays - Nuclear proteins were isolated from stimulated and control cells by scraping them off of culture dishes in ice cold phosphate buffered saline followed by centrifugation for 10 seconds in a microcentrifuge (4°C). The supernate was discarded and the pellet resuspended in 400 μl of swelling buffer (10 mM Hepes-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF). Following a 10 min incubation on ice, cells were vortexed and pelleted in a microcentrifuge (4°C). The supernate was discarded and the pellet was resuspended in 100 μl of resuspension buffer (20 mM Hepes-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF). Following a 20 min incubation on ice, cells were pelleted for 2 min in a microcentrifuge (4°C). The supernate was removed and used in electromobility shift assays (EMSAs) following determination of protein content using a Bradford assay (30). These nuclear extracts (25 μg) were incubated with 20 μg/ml poly dI•dC, 50 μM pyrophosphate and 40,000 cpm of a γ-^{32}P-ATP end-labelled oligonucleotide (containing the sequence between +75 and +104 bp of the cytochrome c gene) in a binding buffer (20 mM Tris pH 7.6, 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine, 1 mM EDTA) at room temperature for 20 min. To determine the specificity of binding, competition assays were conducted by pre-incubating

extracts (20 min) with a 100 molar excess of cold oligonucleotide before the addition of labelled oligonucleotide. The oligonucleotides used in these competition assays were comprised of 1) +75 to +104 bp of the first intron of the cytochrome c gene, 2) the consensus Sp1 binding site, and 3) the consensus Egr-1 binding site. Samples were run on a non-denaturing 4 % acrylamide gel and were electrophoresed for 3 hours (200 V). The gel was subsequently fixed for 15 minutes in acetic acid/methanol/H_2O (10:30:60), dried and imaged utilizing an Instantimager (Packard).

Immunoblotting - C2C12 muscle cells were rinsed in ice-cold PBS and subsequently scraped from culture dishes in 200 µl of 1 X Laemmli buffer (62.5 mM Tris, 20% glycerol, 2% SDS and 5 % 2-mercaptoethanol). Equal amounts of nuclear extracts (50 µg) were size-separated by electrophoresis on a 12 % SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes and probed with polyclonal antibodies for Egr-1, Sp1 or cyclin D1 (1:500). Blots were then probed with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1000) and visualized with an enhanced chemiluminescence kit (Amersham-Pharmacia Biotech, Baie d' Urfé, PQ, Canada).

Statistical Analyses - The data presented are means ± S.E.M. One-way analyses of variance (ANOVAs) were used to evaluate the effects of contractile activity, alone or in combination with the overexpression of wt or mt Egr-1, on cytochrome c mRNA levels and transcriptional activation. One-way ANOVAs were also used to measure the effects of TTX, BAPTA-AM and BDM on cytochrome c transcriptional activation in stimulated and non-stimulated control cells. In all cases individual differences were determined using *Tukey's*

post-hoc test and differences were considered significant if $p<0.05$.

RESULTS

Effectiveness of Stimulation of C2C12 Muscle Cells - Using our cell culture model, myotubes were observed to contract synchronously and at the correct frequency (5 Hz) using voltages as low as 35 V. Cells appeared to reach maximal shortening at 55 V with no further increase in contraction intensity apparent at voltages as high as 110 V. Thus, cells in subsequent experiments were stimulated at an intensity of 65 V ($1.2 V/cm^2$). This voltage is much lower than that previously used to stimulate cardiac myocytes in culture (31, 32) and resulted in minimal myotube detachment following up to 8 h of continuous stimulation[2].

Egr-1 mRNA is Increased Following Electrical Stimulation - Increases ($p<0.05$) in egr-1 mRNA levels were evident as early as 15 minutes after the onset of stimulation in C2C12 myocytes (Fig. 1*A*). This stimulation-induced increase in egr-1 mRNA was transient in nature and reached a maximum of 4.0-fold above that in non-stimulated control cells following 30 min of contractile activity and declined to 2.0-fold above control after 4 h of stimulation. This effect of contractile activity on egr-1 expression is similar to that observed previously *in vivo* (19) and it demonstrates the effectiveness of the myocyte electrical stimulation model in rapidly altering muscle gene expression. The result also suggests the involvement of Egr-1 in the activity-induced adaptations which occur in skeletal muscle.

Electrical Stimulation and Forced Overexpression of Egr-1 Increase Cytochrome c mRNA Levels - Cytochrome c was chosen as a representative model of the effect of

contractile activity on the expression of nuclear genes encoding mitochondrial proteins. In addition to electrical stimulation (5 Hz, 65 V, 3 h/day, 4 days), muscle cells were forced to overexpress either a wild-type (wt) Egr-1 protein or a mutant (mt) form of Egr-1 lacking a portion of the DNA-binding domain. Electrical stimulation resulted in an elevation in cytochrome c mRNA to levels which were 1.6-fold above those in non-stimulated cells (Fig. 1*B*, *lanes 1-2*, $p<0.05$). This adaptation occurred prior to changes in mitochondrial enzymes, reflected by cytochrome c oxidase activity which was 157.3 ± 20.1 nmol/min/mg and 150.0 $\pm$ 2.7 nmol/min/mg in control and stimulated cells, respectively. Transfection of non-stimulated cells with wt Egr-1 resulted in a 2.2 ± 0.4-fold increase in the levels of the protein. Coincident with this was an observed increase in cytochrome c mRNA level which was 2.2-fold higher ($p<0.05$) than that found in quiescent cells transfected with an empty vector (C + ev; Fig. 1*B*). Electrical stimulation of myocytes overexpressing wt Egr-1 led to a further increase ($p<0.05$) in cytochrome c mRNA levels, reaching values which were 3.6-fold above that measured in C + ev cells (Fig. 1*B*). Overexpression of mt Egr-1 had no effect on cytochrome c mRNA, regardless of whether the cells were quiescent or subjected to 4 days of electrical stimulation (Fig. 1*B*).

Electrical Stimulation of C2C12 Muscle Cells Increases Cytochrome c Transcriptional Activation - In order to assess whether electrical stimulation affects cytochrome c transcription, myocytes were transfected with pRC4CAT/-726 plasmid constructs which contained the full-length cytochrome c promoter linked to a CAT reporter gene. CAT assays revealed a 1.5-fold higher ($p<0.05$) cytochrome c transcriptional activation in stimulated,

compared to non-stimulated control cells (Fig. 2*A*), which paralleled the stimulation-induced increase in cytochrome c mRNA (Fig. 1*B*). Since it has been shown previously that the factors involved in Ca^{2+}-mediated increases in transcription act within the minimal -66 bp cytochrome c promoter (7), we transfected cells with plasmid constructs containing this region (pRC4CAT/-66). Electrical stimulation of these cells elicited a 1.7-fold higher ($p<0.05$) CAT activity compared to non-stimulated control cells (Fig.2*B*). This response mirrored Ca^{2+}-mediated expression of cytochrome c and suggested the involvement of Ca^{2+} in the activity-induced expression of cytochrome c. Furthermore, the element responsible for the activity-induced transactivation of the cytochrome c gene is likely located within this minimal promoter.

Egr-1-mediated Cytochrome c mRNA Expression Involves mRNA Stability and is Independent of Direct Transcriptional Activation - The contribution of Egr-1 to the activity-induced cytochrome c transcriptional activation was assessed by transfecting cells with wt Egr-1 in combination with the -726 bp cytochrome c promoter. Overexpression of wt Egr-1 in quiescent cells had no effect on cytochrome c transcriptional activation (Fig. 2*A*) despite a 2.2-fold elevation in cytochrome c mRNA under similar conditions. In addition, subjecting these myocytes to contractile activity elicited no effect on cytochrome c transcriptional activation (Fig. 2*A*). Similar results were obtained in cells transfected with mt Egr-1 (Fig. 2*A*). When myocytes were transfected with the minimal -66 bp cytochrome c promoter (pRC4CAT/-66), no effect of the overexpression of wt or mt Egr-1 on CAT activity was evident either in the presence or absence of electrical stimulation (Fig. 2*B*). Thus, Egr-1

appears to increase the expression of cytochrome c mRNA independent of direct transcriptional activation, suggesting a role for Egr-1 in the regulation of cytochrome c mRNA stability. To evaluate this possibility, we measured cytochrome c degradation in stimulated and non-stimulated cells that were transfected with either ev or wt Egr-1 plasmids. Similar degradation rates of cytochrome c mRNA were observed in stimulated and control cells that were transfected with ev, which declined by 23.8 ± 4.5 and 30.1 ± 9.7%, respectively, after 48 hours of transcriptional inhibition with ACT (Fig. 2*C*). Cytochrome c mRNAs were very stable in these cells, exhibiting an extrapolated half-life of approximately 89 hours. In contrast, no reductions in cytochrome c mRNA levels were evident following 48 hours of ACT treatment in stimulated or non-stimulated cells that were overexpressing Egr-1 (Fig. 2*C*). This illustrates a role for Egr-1 in the regulation of cytochrome c mRNA stability.

Sp1 binding within the first intron of the cytochrome c promoter is increased by contractile activity - Following four days of electrical stimulation (3 h/day) there is a contractile activity-induced 2.4 ± 0.5-fold increase in Sp1 protein levels, while no effect of electrical stimulation on Egr-1 protein levels was evident (Fig 3*A*). Coincident with this increase in Sp1 protein levels was an 1.8 ± 0.2-fold increase in DNA binding within the first intron (+75 to +104 bp) of the cytochrome c promoter (Fig. 3*B lane 2 versus 3*). This binding is prevented by incubation with 100-molar excess of a cold oligodeoxynucleotide containing a portion of the first intron of the cytochrome c gene (Fig. 3*B lane 4*). In addition, DNA binding was eliminated by pre-incubation with a non-radiolabelled consensus

Sp1 deoxyribonucleotide (Fig. 3*B*, *lane 5*), suggesting that Sp1 may be responsible for the activity-induced activation of cytochrome c transcription. There was no effect of pre-incubation with a cold oligonucleotide containing the consensus Egr-1 binding sequence (Fig. 3*B*, *lane 6*). To further evaluate the effects of contractile activity on Sp1 activation, extracts from stimulated and control cells were incubated with a radiolabelled consensus Sp1 sequence (Fig. 3*C*). These analyses revealed that there was a stimulation-induced elevation in Sp1 DNA binding (Fig. 3*C*, *lane 2 versus 3*), which was completely eliminated by the addition of an excess of cold Sp1 consensus oligonucleotide (Fig. 3*C*, *lane 5*). In contrast, there was no effect of contractile activity on binding to an Egr-1 consensus oligonucleotide (Fig. 3*C*, *lanes 8,9*). Egr-1 binding was not evident in the presence of an excess of either cold Sp1 or Egr-1 consensus oligonucleotides (Fig. 3*C*, *lanes 11,12*), while no effect of pre-incubation with cold -66 oligonucleotide on binding to the Egr-1 consensus sequence was observed (Fig. 3*C*, *lane 10*). Taken together, these results suggest that electrical stimulation increases Sp1 binding within the first intron of the cytochrome c gene and that Egr-1 does not bind within this region. In addition, there may be an interaction between Egr-1 and Sp1, which may be an important factor in the elimination of activity-induced transcriptional activation cytochrome c when Egr-1 is overexpressed (Figs. 2*B* and 2*C*).

Cross-bridge Cycling is Necessary for Activity-induced Transcriptional Activation of Cytochrome c - To evaluate the event occurring during contractile activity which provides the signal responsible for activity-induced cytochrome c transactivation, muscle contraction was inhibited at various levels. Cells were transfected with the -726 bp cytochrome c

promoter and were treated 24 h prior to the onset of stimulation with 1) TTX (10 μM) to prevent membrane depolarization; 2) BAPTA-AM (25 μM) to prevent the increase in $[Ca^{2+}]_{ic}$ associated with Ca^{2+} release from the sarcoplasmic reticulum; or 3) BDM (1.5 mM) to prevent cross-bridge cycling. Pre-treatment of control cells with TTX abolished muscle contraction entirely but had no effect on basal levels of cytochrome c transcriptional activation compared to VEH-treated cells (Fig. 4*A*). Contractile activity induced a 1.8-fold activation ($p<0.05$) of the -726 bp cytochrome c promoter in the presence of VEH and this effect was completely prevented by TTX treatment (Fig. 4*A*). BAPTA-AM (25 μM) was used to permit membrane depolarization while preventing stimulation-induced muscle shortening via the chelation of intracellular Ca^{2+}. In this situation, incubation with BAPTA-AM (25 μM) resulted in complete inhibition of muscle contraction. BAPTA-AM treatment resulted in a 37 % decrease ($p<0.05$) in cytochrome c transcriptional activation in non-stimulated cells compared to VEH treated cells (Fig. 4*B*), suggesting the involvement of Ca^{2+} in cytochrome c transcription, as described recently (7). Myotubes subjected to electrical stimulation demonstrated a 1.5-fold increase ($p<0.05$) in cytochrome c transactivation above that in non-stimulated control cells, while stimulated cells pre-treated with BAPTA-AM showed no elevation in cytochrome c transcriptional activation above that found in C + VEH cells (Fig. 4*B*). Inhibition of contractile activity at the level of cross-bridge cycling with BDM was employed to allow membrane depolarization and $[Ca^{2+}]_{ic}$ cycling to occur in response to electrical stimulation, while preventing sarcomere shortening. BDM had no effect on cytochrome c transcriptional activation in non-stimulated cells (Fig.

4*C*). Electrical stimulation of VEH-treated cell resulted in a 1.4-fold elevation ($p<0.05$) in cytochrome c transcriptional activity while treatment of these cells with BDM prevented this contractile activity-induced elevation in cytochrome c transcription (Fig. 4*C*).

DISCUSSION

Contractile activity is a potent stimulus for the induction of numerous cellular adaptations in skeletal muscle, including mitochondrial biogenesis (1, 33). This complex process involves the coordinated expression of proteins encoded within the nucleus as well as those encoded in mitochondrion, suggesting that a vital intracellular communication exists between these two organelles. It is likely that mitochondrial assembly is regulated mainly by proteins originating from the nucleus, since most of the factors required for the expression of mitochondrial proteins are nuclear-encoded, and the vast majority of the proteins found within the organelle are derived from the nuclear genome. We have adopted the respiratory chain protein cytochrome c as a model for the regulation of nuclear gene expression in response to contractile activity. Recently, we demonstrated that an elevation in contractile activity evokes time-dependent augmentations in cytochrome c transcriptional activation *in vivo* (5). Here our goal was to utilize a cell culture model of contractile activity in order to mimic the *in vivo* induction of cytochrome c transcription, while allowing for the definition of some of the intracellular signals and transcription factors responsible for this adaptation.

Electrical stimulation of C2C12 cells in culture elevated the contractile activity of differentiated myotubes, which was clearly visible upon inspection under the microscope compared to non-stimulated control cells. Contractile activity imposed for 4 days (3 hrs/day) induced an elevation in cytochrome c mRNA, which was parallelled by alterations in gene transcription. In order to define more precisely the intracellular events associated with muscle contraction which mediated this transcriptional response, muscle contraction was

systematically disrupted at three different levels. Treatment of cells with TTX completely abolished muscle contraction by preventing membrane depolarization, which should eliminate the subsequent release of Ca^{2+} and actin-myosin interactions. As expected, this prevented the activity-induced cytochrome c transcriptional activation which was apparent in vehicle-treated cells. Next, the role of Ca^{2+} in activity-induced cytochrome c expression was evaluated using the membrane-permeable Ca^{2+} chelator BAPTA-AM. The potential involvement of intracellular Ca^{2+} in activity-induced cytochrome c expression was suggested from observations that Ca^{2+} serves as a potent intracellular second messenger for a variety of cellular adaptations (10, 34, 35). In addition, we recently reported that a marked elevation in cytochrome c transcriptional activation occurs following the treatment of rat L6E9 muscle cells with the Ca^{2+} ionophore A23187 (7), an effect which is reproducible in the mouse C2C12 muscle cells used in the present study[2]. Treatment with BAPTA-AM should permit the transmission of membrane depolarization, but eliminate sarcoplasmic reticulum-mediated Ca^{2+} transients and subsequent actin-myosin interactions. The complete inhibition of contractile activity which we observed during these conditions was also associated with the abolition of the simulation-induced cytochrome c transcriptional activation. This suggests that membrane depolarization alone, leading to voltage-sensitive activation of signalling cascades which are known to activate the transcription of numerous genes in excitable cells (cf. ref. 5 for review), cannot account for the observed increases in cytochrome c transactivation. Finally, we evaluated contractile activity-induced increases in cytochrome c transactivation in the presence and absence of BDM, an agent known to eliminate cross-

bridge cycling, and presumably ATP utilization, but allow both membrane depolarization and the increase in Ca^{2+} transients to occur. We hypothesized that if Ca^{2+} was a primary intracellular signal mediating the increase in muscle cytochrome c transactivation (7), this transcriptional activation should be evident even when muscle contraction was inhibited in electrically stimulated cells by preventing cross-bridge cycling. However, under these conditions no elevation in cytochrome c transcription was evident, suggesting that cross-bridge cycling, and attendant ATP utilization, represent more important signals for contractile activity-induced mitochondrial biogenesis. Interestingly, other studies in cardiac muscle have also shown that cross-bridge formation and subsequent muscle shortening are vital for phenylepherine-induced adaptations in myosin expression (9). However, it still remains unclear as to which element of cross-bridge cycling is responsible for the initiation of cytochrome c transcription, and potentially for mitochondrial biogenesis in general.

Previous studies have shown that the transcriptional activation of cytochrome c in response to elevated intracellular Ca^{2+} are mediated by factors which bind to elements within the minimal (-66 to +115 bp) promoter region of the cytochrome c gene (7). Our data also indicate that this region is responsive to contractile activity (Figs. 3*B*, 4*A*). Within the first intron of the cytochrome c gene are GC-rich regions which may serve as binding sites for the zinc-finger transcription factors Sp1 and Egr-1 (13, 14). Indeed, Egr-1 mRNA levels were elevated very rapidly after the onset of muscle contraction (Fig. 1*A*) and overexpression of this protein led to an augmentation of cytochrome c mRNA in stimulated and non-stimulated cells. These results suggested that Egr-1 was activated in response to contractile activity and

could possibly be involved in the elevation of cytochrome c transcription. However, additional studies showed that the overexpression of Egr-1 elicited no increase in cytochrome c transactivation (Figs. 3 *A* and 3*B*). Unexpectedly, we found that Egr-1 overexpression led to a significant stabilization of cytochrome c mRNA (Fig. 2*C*). This suggests that Egr-1 may be involved in the transcriptional activation of a stabilizing factor, possibly the AU-rich element binding protein AUF1 (36), which is involved in the regulation of cytochrome c mRNA stability. The rapid onset of egr-1 mRNA is consistent with the early stabilization of cytochrome c mRNA which we have observed to occur in response to chronic contractile activity of rat tibialis anterior muscle (5).

It is known that Sp1 is involved in the expression of a variety of mammalian genes involved in oxidative phosphorylation (37). In particular, Sp1 affects cytochrome c expression by binding within the region between +83 and +104 bp of the gene (15). Here we show that contractile activity increases Sp1 protein levels and causes an elevation in DNA-binding within this region and to a consensus Sp1 oligonucleotide, and in both cases DNA-binding is completely eliminated by pre-incubation with a non-labelled Sp1, but not an Egr-1, oligonucleotide (Figs. 4*A*, 4*B*). Thus, it appears that contractile activity induces cytochrome c transcription via mechanisms that involve Sp1. It is interesting that Egr-1 overexpression results in a removal of the contractile activity-induced cytochrome c transcriptional activation, suggesting that elevated levels of Egr-1 interfere with the normal Sp1-mediated effect. This could occur because of interactions between the two proteins, a possibility that has recently been established (17, 18). Inhibition of the contractile activity-

induced cytochrome c transactivation by the mutant form of Egr-1, which is incapable of binding DNA, further suggests that this effect may be a result of a direct Sp1-Egr-1 protein interaction. The ubiquitous nature of Sp1 makes it a likely target for regulation by numerous cellular proteins, and it has been recently shown that histone deacetylase, a protein seemingly unrelated to Sp1, serves to repress Sp1 function (38). However, more work is necessary to determine the scope of the possible interactions between Egr-1 and Sp1 in skeletal muscle cells.

In summary, it appears from the data presented here that ATP-dependent cross-bridge cycling provides a putative signal which induces skeletal muscle to initiate the transcriptional activation of nuclear-encoded mitochondrial genes. The levels of the mRNAs encoding Egr-1 are rapidly increased in response to electrical stimulation, and induction of the protein may represent the initial steps in the stabilization of nuclear-encoded mRNAs encoding mitochondrial proteins. The *cis*-elements between +75 and +104 bp relative to the transcription start site of the cytochrome c gene appear to be partly responsible for the contractile activity-induced transcriptional activation of cytochrome c, and this response appears to be reliant on enhanced Sp1 DNA-binding. Finally, the contractile activity-induced transcriptional activation of cytochrome c can be inhibited by overexpression of Egr-1, suggesting a repressive role for this protein on cytochrome c transcription, possibly via protein-protein interactions with Sp1.

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FIG. 1. **Effect of contractile activity on egr-1 and cytochrome c mRNA levels in C2C12 myocytes.** *A,* Cells were electrically stimulated (5 Hz, 65 V) for 5, 15, 30, and 60 min and harvested immediately following the cessation of stimulation. Total RNA was extracted and Northern blot analyses were carried out as described in "Experimental Procedures". A typical Northern blot showing the levels of egr-1 mRNA in electrically stimulated cells (S) compared to non-stimulated control cells (C) is shown (*inset*) and the results of repeated experiments are depicted graphically (*, $p<0.05$ *versus* control). *B*, C2C12 myoblasts were transfected with 5 μg of either pCMV/930 (wt), pCMV/858 (mt) or an empty vector (ev). Cells were then electrically stimulated (5 Hz, 65 V) for 4 days (3 h/day) or remained quiescent for a similar time period. Total RNA extraction and Northern blot analyses were carried out as described in "Experimental Procedures". Autoradiograms demonstrating the levels of cytochrome c mRNA (*inset*) in C and S cells transfected with ev, wt or mt plasmids were quantified by electronic autoradiography. (*, $p<0.05$ *versus* C + ev; ¶, $p<0.05$ *versus* all other groups). Egr-1 and cytochrome c mRNA levels were corrected for uneven loading with 18S rRNA and are expressed as a percentage of the levels observed in control cells. Values are mean ± S.E.M..

FIG. 2. **Effects of contractile activity and Egr-1 on transcriptional activation of the full-length and minimal cytochrome c promoters in skeletal muscle cells.** *A,* C2C12 myoblasts were transfected with 5 μg of either pCMV/930 (wt), pCMV/858 (mt) or an empty vector (ev), 5 μg of pRC4CAT/-726 and 5 μg of pRSV/β-gal. Cells were then electrically stimulated (5 Hz, 65 V) for 4 days (3 h/day) or remained quiescent for a similar time period. CAT and β-galactosidase activities were measured as described in "Experimental Procedures". Autoradiograms of CAT assays (*inset*) from control cells (C) and electrically stimulated cells (S) transfected with ev, wt or mt plasmids were quantified by electronic autoradiography. (*, $p<0.05$ *versus* C + ev). Values are mean ± S.E.M. of at least 7 independent experiments. *B,* To determine the effects of stimulation and Egr-1 overexpression on activation of the minimal cytochrome c promoter, cells were transfected as in (*A*) but with the minimal promoter pRC4CAT/-66 used instead of pRC4CAT/-726. Autoradiograms of CAT assays (*inset*) from C and S cells transfected with ev, wt or mt Egr-1 were quantified by electronic autoradiography. (*, $p<0.05$ *versus* C + ev). Values are mean ± S.E.M. of at least 6 independent experiments. *C,* Degradation of cytochrome c mRNA in S and C cells expressing ev or wt plasmid DNA was measured following treatment with 10 μg/ml actinomycin D (ACT). RNA was isolated at 4, 24 and 48 hours after the addition of ACT, and cytochrome c mRNA levels were measured and expressed as a percentage of the t=0 value. Values are means ± S.E.M. of 3-4 experiments.

FIG. 3. **The effects of contractile activity on DNA binding within the first intron of the cytochrome c gene.** *A*, C2C12 muscle cells were electrically stimulated (3 h/day, 4 days) and nuclear proteins were isolated as described in "Experimental Procedures". Nuclear extracts (50 μg) from stimulated (S) and control (C) cells were separated by SDS-PAGE and probed with polyclonal antibodies for Sp1, Egr-1 or cyclin D1. *B*, Nuclear proteins (25 μg) from C (*lane 2*) and S cells (*lanes 3-6*) were incubated with a ^{32}P-labelled oligodeoxynucleotide corresponding to +75 to +104 bp of the first intron of the cytochrome c gene. Nuclear extracts were also pre-incubated with non-radiolabelled deoxyoligonucleotides corresponding to this region (-66, *lane 4*), the Sp1 consensus binding site (Sp, *lane 5*) or the Egr-1 consensus binding sequence (Egr, *lane 6*). *C*, Cell extracts (25 μg) from C (*lanes 2, 8*) and S (*lanes 3-6, 9-12*) cells were incubated as in *A* with radiolabelled Sp1 (lanes 1-6) or Egr-1 (*lanes 7-12*) consensus oligonucleotides. Competition reactions were conducted with 100-molar excess non-radiolabelled -66 (*lanes 4, 10*), Sp (*lanes 5, 11*) or Egr (*lanes 6, 12*) oligonucleotides. (FP, free probe).

FIG. 4. **The roles of membrane depolarization, intracellular Ca^{2+} levels and cross-bridge cycling in activity-induced cytochrome c transcriptional activation.** *A*, C2C12 myoblasts were transfected with 5 μg of pRC4CAT/-726 and 5 μg of pRSV/β-gal. Cells were treated with 10 μM TTX, to prevent opening of voltage-gated Na^+ channels, or vehicle (V) 24 h prior to the onset of stimulation. Cells either remained quiescent (C) or were electrically stimulated (S) for 4 days (5 Hz, 65 V, 3 h/day). CAT and β-galactosidase activities were measured as described under "Experimental Procedures". CAT activities observed in C + V cells were set at 100 %, and all other activities were expressed as a percentage of this value. Values are means ± S.E.M. of 6 experiments. *B*, Myoblasts were transfected with 5 μg of pRC4CAT/-726. To prevent the contraction-induced elevation in $[Ca^{2+}]_{ic}$ cells were treated with 25 μM BAPTA-AM or V 24 hours prior to the onset of stimulation. CAT activities were measured as in *A* and expressed as a percentage of the C + V value. Values are means ± S.E.M. of 5 separate experiments. *C*, Myoblasts were transfected with 5 μg of pRC4CAT/-726 and subsequently treated with 1.5 mM BDM, to prevent the cross-bridge cycling, or V 24 hours prior to the onset of stimulation. CAT activities were measured as in *A* and expressed as a percentage of the C + V value. Values are means ± S.E.M. of 5 independent experiments (*, $p<0.05$ *versus* C + V).

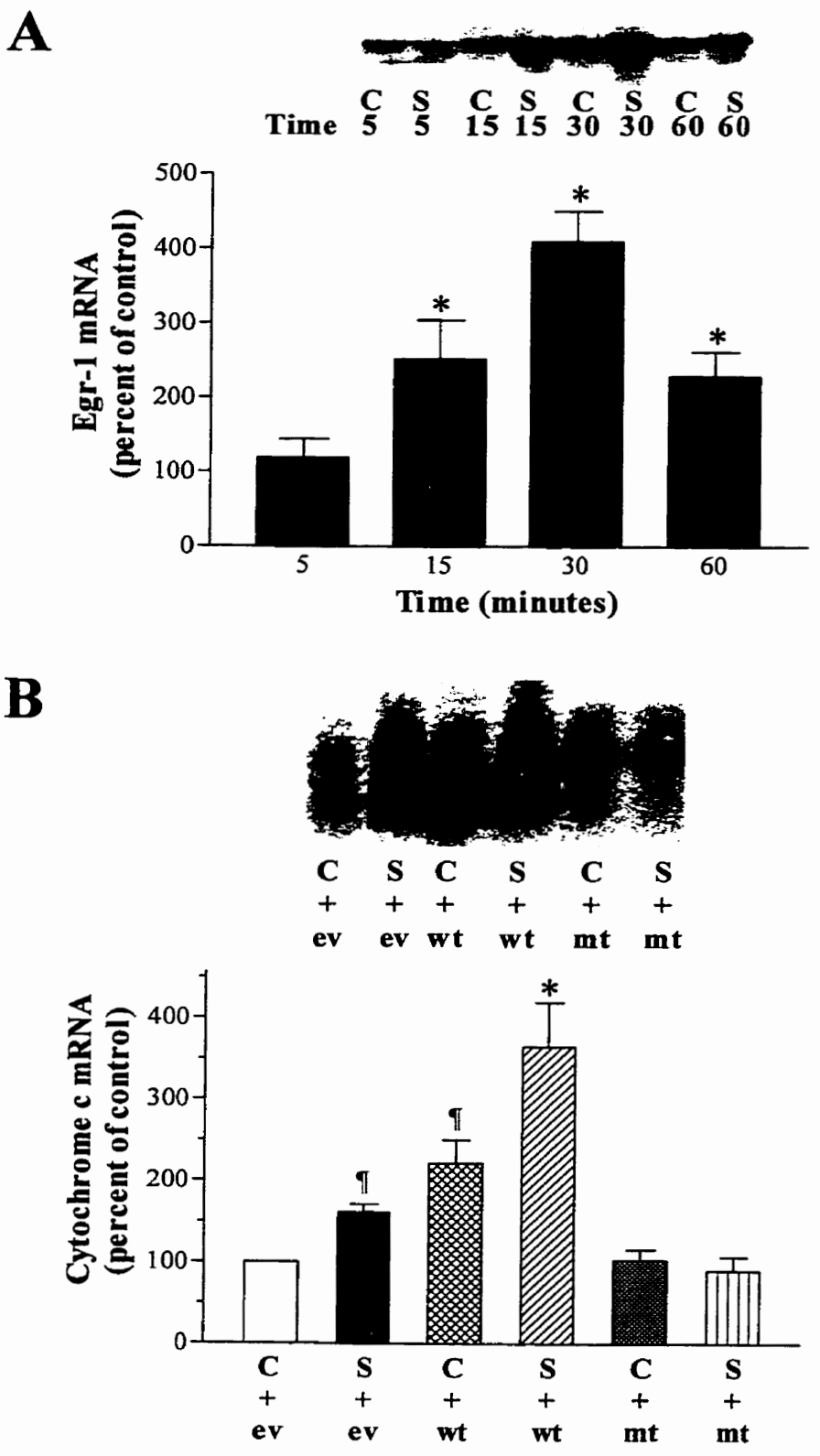
A
C S C S C S C S
Time 5 5 15 15 30 30 60 60
Egr-1 mRNA (percent of control)
500
400
300
200
100
0
*
*
*
5
15
30
60
Time (minutes)
B
C S C S C S
+ + + + + +
ev ev wt wt mt mt
Cytochrome c mRNA (percent of control)
400
300
200
100
0
¶
¶
*
C S C S C S
+ + + + + +
ev ev wt wt mt mt

Figure 1

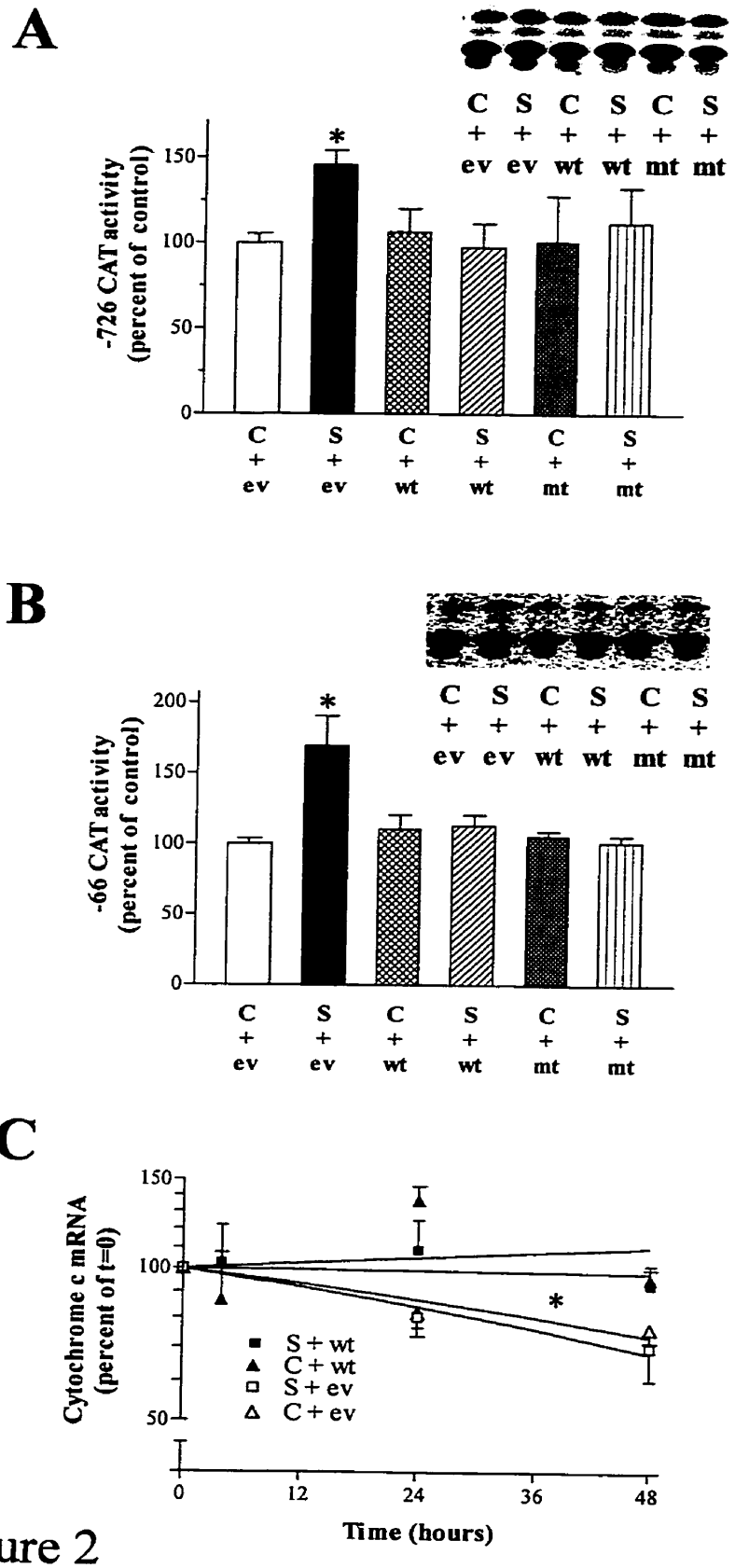
A
C S C S C S
+ + + + + +
ev ev wt wt mt mt
-726 CAT activity
(percent of control)
150
100
50
0
*
C + ev
S + ev
C + wt
S + wt
C + mt
S + mt
B
C S C S C S
+ + + + + +
ev ev wt wt mt mt
-66 CAT activity
(percent of control)
200
150
100
50
0
*
C + ev
S + ev
C + wt
S + wt
C + mt
S + mt
C
Cytochrome c mRNA
(percent of t=0)
150
100
50
*
S + wt
C + wt
S + ev
C + ev
0
12
24
36
48
Time (hours)

Figure 2

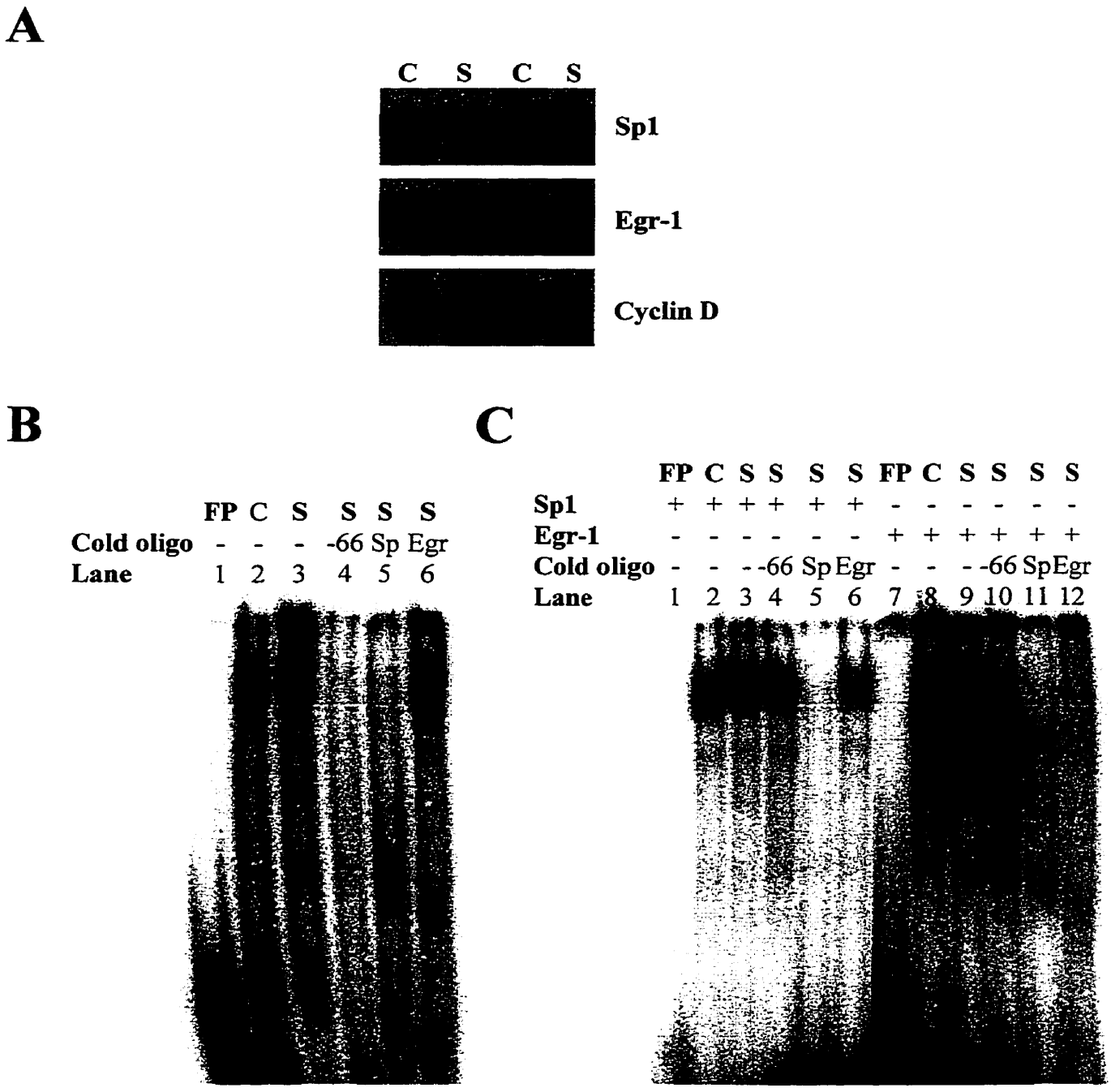
A
C S C S
Sp1
Egr-1
Cyclin D
B
FP C S S S S
Cold oligo - - - -66 Sp Egr
Lane 1 2 3 4 5 6
C
FP C S S S S FP C S S S S
Sp1 + + + + + + - - - - - -
Egr-1 - - - - - - + + + + + +
Cold oligo - - - -66 Sp Egr - - - -66 Sp Egr
Lane 1 2 3 4 5 6 7 8 9 10 11 12

Figure 3

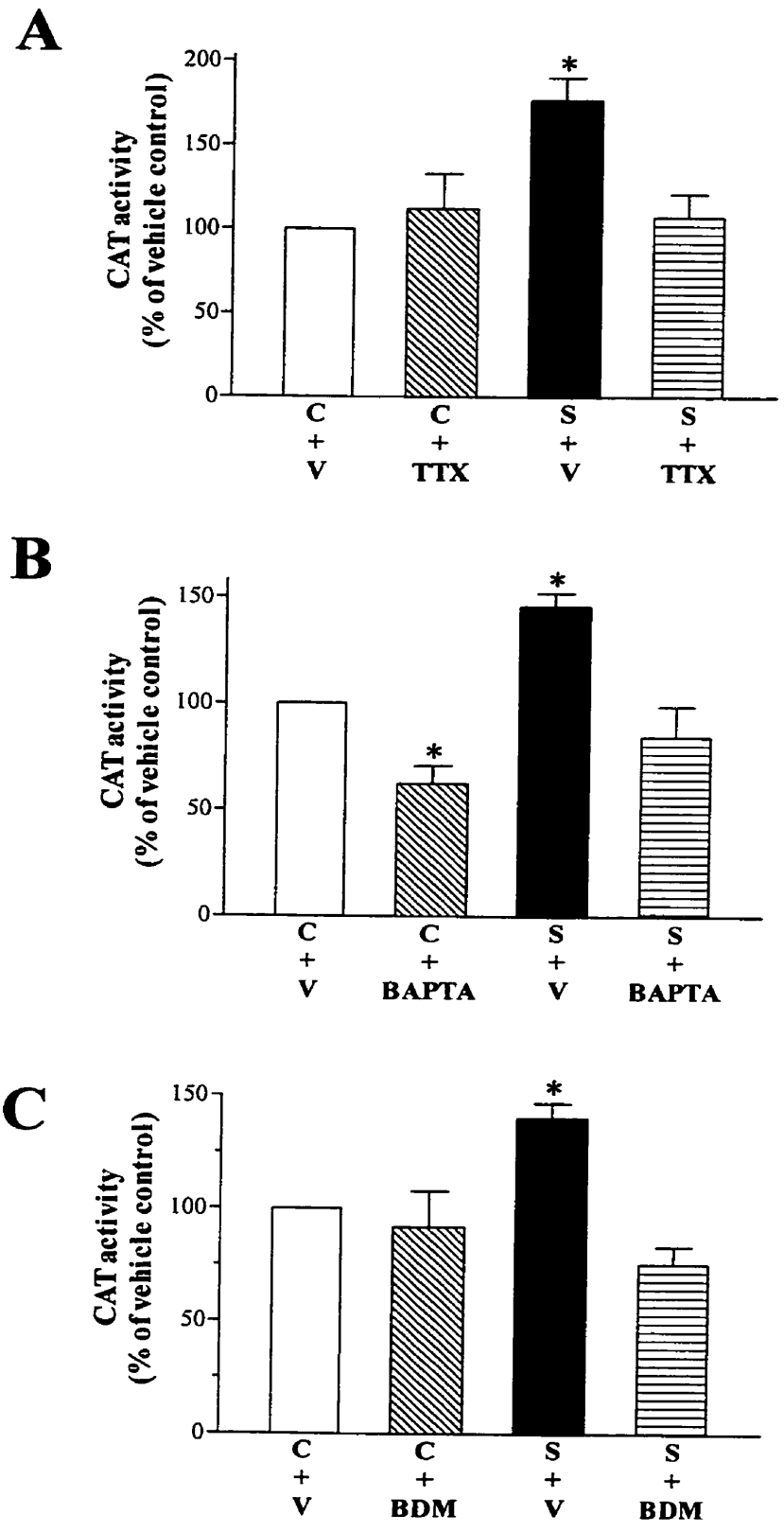
A
CAT activity
(% of vehicle control)
200
150
100
50
0
*
C + V
C + TTX
S + V
S + TTX
B
CAT activity
(% of vehicle control)
150
100
50
0
*
*
C + V
C + BAPTA
S + V
S + BAPTA
C
CAT activity
(% of vehicle control)
150
100
50
0
*
C + V
C + BDM
S + V
S + BDM

Figure 4

GENERAL DISCUSSION

Skeletal muscle is a phenotypically dynamic tissue in which gene expression is driven in part by contractile activity. For example, slow-twitch, postural muscles (i.e. soleus) that are continually active for a large portion of the day in order to maintain body position, have a high mitochondrial content in order to fulfill the large metabolic demand that is placed on the muscle. Without this type of parallellism between contractile activity and the expression of mitochondrial proteins, the muscle would be unable to meet daily energy requirements, which would almost certainly lead to a functional impairment. The majority of mammalian skeletal muscles are mixed muscles consisting of a combination of fast-twitch (red and white) and slow-twitch (red) fibers which serve ambulatory, as opposed to postural functions. As such, these muscles do not demonstrate the sustained high levels of activity that those muscles comprised mainly of slow-twitch fibers do, and therefore exhibit lower mitochondrial protein levels.

Fast-twitch skeletal muscles can adapt their phenotype in response to alterations in contractile activity, and given the appropriate stimulus, muscles such as the tibialis anterior can markedly increase mitochondrial protein content to levels similar to those in the highly oxidative soleus muscle (Ornatsky et al., 1995). Although alterations in skeletal muscle gene expression in response to increased contractile activity have been well documented (Essig, 1996; Pette and Vrbová, 1992), the mechanisms central to these adaptations remain relatively unknown. In addition to increased contractile activity, skeletal muscle gene expression can change in response to decreases in activity. In general, upon reductions in contractile activity

skeletal muscles will tend to rely less on aerobic means for the provision of energy, as indicated by an increase in glycolytic capacity and a general reduction in the activity of the enzymes responsible for aerobic metabolism per gram of tissue (Thomason and Booth, 1990). However, this response does not appear to be universal, as some models of decreased muscle use induce different adaptations. For example, denervation causes a reduction in the activity of mitochondrial enzymes (Eisenberg and Hood, 1993: Jasmin et al., 1995; Wicks and Hood, 1991) and mitochondrial volume (Lu et al., 1997), a consistent response based on the enzyme activity changes that during increases in muscle contraction (Pette and Vrbová, 1992). However, upon exposure to microgravity, there are decreases in the levels of some mitochondrial enzymes while others remain unchanged (Baldwin, 1996; Roy et al., 1996). Thus, there appears to be model-specific effects that underlie these discrepancies in response.

The current literature on the effects of microgravity on skeletal muscle gene expression demonstrates an inconsistent response to this stimulus, and no study has examined gene expression in the heart following spaceflight. To address this, the first study (Manuscript#1) examined the effects of short-term (6 days) exposure to microgravity, as elicited by spaceflight, on the expression of mitochondrial proteins in skeletal and cardiac muscle. Cytochrome c oxidase (CYTOX) enzyme activity was reduced in the biceps of animals exposed to microgravity, which was accompanied by an increase in the activity of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) while no change in malate dehydrogenase (MDH) activity was evident. This response suggested that the muscle was becoming more reliant on glycolytic metabolism for the provision of ATP. The

decrease in CYTOX activity in the triceps occurred in the absence of changes in the levels of the mRNAs encoding CYTOX subunits III, IV and VIc, suggesting that microgravity elicited an increased skeletal muscle protein turnover, which in turn affected holoenzyme function. In addition, despite these changes in CYTOX activity there was no effect of spaceflight on triceps subunit IV protein levels. Thus, if an increase in protein turnover indeed occurs in skeletal muscle during spaceflight it does not appear to be universal in scope. In addition, the disproportionate response of subunit IV protein levels and CYTOX enzyme activity suggests that this is not the rate limiting subunit, and that changes in enzyme activity may reflect impaired holoenzyme assembly.

Little information is known on the effects of spaceflight on the cardiovascular system, and no study has examined the effect of microgravity on the expression of mitochondrial proteins in cardiac muscle. Microgravity-induced elevations in MDH enzyme activity, which were accompanied by changes in MDH mRNA levels in the heart. Similarly, spaceflight induced modest increases (mean = 30%) in the levels of cardiac CYTOX subunit III, IV and VIc mRNAs while CYTOX subunit IV protein levels were doubled. Thus, these data cumulatively suggested that the heart was undergoing mitochondrial biogenesis. However, no changes in CYTOX activity were evident, despite adaptations in the expression of the mRNAs encoding specific enzyme subunits. Thus, cardiac and skeletal muscle appear to adapt differently to microgravity. Skeletal muscle appears to be moving towards a reliance on glycolysis for the provision of energy, while the heart is attempting to increase the expression of mitochondrial proteins. In addition, there may be a disregulataion in the

expression of mitochondrial genes, which is suggested by the lack of coordination between the response of mRNA and protein levels to microgravity.

The expression of genes encoding mitochondrial proteins in skeletal muscle in response to elevations in contractile activity has been well documented (Hood et al., 1989; Takahashi et al., 1998; Williams et al., 1986; Yan et al., 1996). However, these studies are mainly descriptive and do not provide direct evidence of the mechanisms behind these adaptations. To address this, experiments were conducted (Manuscripts #2 and #3) which evaluated the response of cytochrome c, a nuclear-encoded mitochondrial protein, to increased contractile activity. It is known that continuous chronic contractile activity increases cytochrome c mRNA levels which are associated with reduced RNA-protein interactions in the 3'-untranslated region (Yan et al., 1996). However, the physiological ramifications of this finding were unknown and direct evidence of alterations in mRNA stability were not established. This is not a trivial undertaking, as the mRNAs which encode skeletal muscle mitochondrial proteins have been shown to be very stable (Connor et al., 1996). This means that mRNA turnover likely occurs at very low rates, making the evaluation of mRNA transcription and degradation difficult using nuclear run-on and transcriptional inhibition, respectively. Therefore, we set out to determine effective methods to analyze these processes using an *in vivo* chronic stimulation model (Manuscript #2). Intermittent contractile activity (3 hours/day) elicited increases in cytochrome c mRNA and protein levels. Interestingly, intermittent bouts of muscle contraction led to a more rapid onset of cytochrome c mRNA augmentations when compared to those elicited by continuous stimulation (Yan et al., 1996).

This substantiates previous observations that events occurring during recovery from contractile activity are important for the induction of adaptations in gene expression (Neufer et al., 1998; Takahashi et al., 1993). By developing an *in vitro* mRNA decay system we illustrated a transient activity-induced elevation in cytochrome c mRNA stability, which was accompanied by elevated cellular mRNA levels. Thus, we originally hypothesized that contractile activity induces a protein which stabilizes cytochrome c mRNA. However, other work has provided more direct evidence that muscle contraction reduces the levels of a destabilizing factor (McClure and Hamilton, 1999;Yan et al., 1996). This adaptation in stability occurred prior to increases in cytochrome c transcription, as measured using direct muscle gene injection (Davis and Jasmin, 1993; Thomason and Booth; 1990). Thus, the use of these techniques allowed us to demonstrate the temporal increases in both cytochrome c mRNA transcription and stability to contractile activity.

It is known that acute muscle contraction can activate multiple signalling pathways (Aronson et al., 1997; Goodyear et al., 1996; Hayashi et al., 1999), but the consequences of this activation on gene expression remain unclear. Thus, we developed a muscle cell culture model to define the potential signals generated by muscle contraction, which may lead to the increased activity of these signalling pathways and subsequently cause alterations in gene expression (Manuscript #3). Membrane depolarization, Ca^{2+} mobilization, cross-bridge cycling and alterations in energy metabolism are events which occur during muscle contraction. Each of these are known to initiate intracellular signalling cascades (Biswas et al., 1999; Eble et al., 1998; Fields et al., 1997; Hardingham et al., 1997; Schmidt, 1995)

which could ultimately lead to altered gene expression. In addition, it has been recently reported that Ca^{2+} activates the transcription of the full-length (-726 to +115 bp) and the minimal (-66 to +115 bp) cytochrome c promoters (Freyssenet et al., 1999). We demonstrated that contractile activity elicits similar adaptations in cytochrome c transcription which suggests that 1) Ca^{2+} may be the component of muscle contraction that initiates alterations in gene expression and 2) the factors that mediate this response bind to the region between -66 to +115 bp of the cytochrome c gene. However, treatment of myocytes with butanedione monoxime, an agent that allows for stimulation-induced increases in membrane depolarization and intracellular Ca^{2+} to occur while preventing cross-bridge cycling, completely abolished muscle contraction and prevented the associated increase cytochrome c gene transcription. Thus, it appears that elements of muscle contraction which occur downstream of the increase in $[Ca^{2+}]_{ic}$, perhaps cross-bridge cycling or the attendant ATP utilization, are responsible for initiating adaptations in cytochrome c gene expression.

Further examination of the cytochrome c minimal promoter revealed potential binding sites for the zinc finger transcription factors Sp1 and Egr-1 (Evans and Scarpulla, 1989), which bind GC-rich DNA sequences (Berg, 1992; Evans and Scarpulla, 1989), making these two proteins prime candidates to mediate the transcriptional activation of cytochrome c. To evaluate this, we transfected cells with an expression vector for Egr-1 which resulted in an increase in cytochrome c mRNA, and this effect was enhanced by electrical stimulation. Surprisingly, Egr-1 overexpression elicited changes in cytochrome c mRNA stability but had no effect on transcriptional activation. We hypothesize that Egr-1 acts to repress the

transcription of a destabilizing factor, leading to an increased mRNA stability (Fig. 1). When we examined DNA binding within the first intron of the cytochrome c gene, there was an activity-induced increase in binding, which was parallelled by an elevated binding to an Sp1, but not an Egr-1, consensus oligonucleotide. Thus, it appears that contractile activity increases DNA binding within the first intron of the cytochrome c gene and that this response may involve Sp1 (Fig. 1). However, it should be noted that these results are not the definitive experiments that show unequivocally that Sp1 alone is responsible for the activity-induced expression of cytochrome c. However, we do provide solid evidence that Sp1 is involved in this response and establish a direction for future experiments utilizing primary skeletal muscle cells.

In summary, we have shown that mitochondrial biogenesis in heart and skeletal muscle responds differently to microgravity. Further, in skeletal muscle it appears that increases in contractile activity elicit sequential, time dependent elevations in the stability and transcription of cytochrome c mRNA, a nuclear gene product. The contraction-induced transcriptional activation of the cytochrome c gene in skeletal muscle appears to involve Sp1, but not Egr-1 binding within the first intron (Fig. 1). There is also evidence that Egr-1 may play a role in altering in cytochrome c mRNA stability following elevations in muscle activity by mediating the expression of a destabilizing factor (Fig.1). Finally, all of these adaptations seem to require ATP-dependent cross-bridge cycling, which may represent a putative cellular signal for the initiation of alterations in skeletal muscle gene expression.

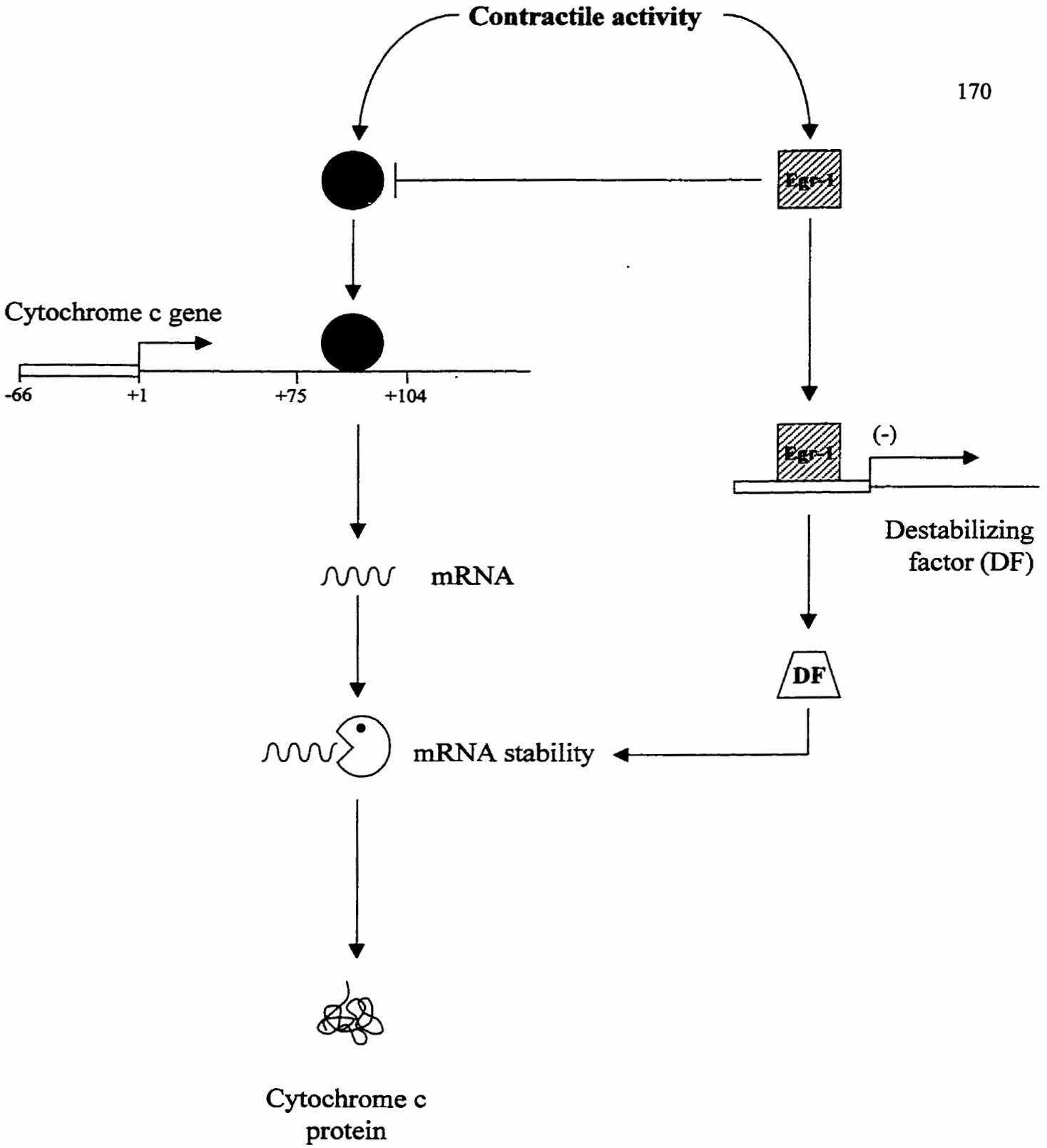

Figure 1. Working hypothesis for the regulation of cytochrome c expression in response to increased contractile activity (see text for details).

Limitations of the Study

In most experimental procedures there are caveats associated with their implementation. Such protocols were used in the final manuscript in my thesis and I will attempt to outline both the rationale and the justifications for using these protocols.

Skeletal muscle contraction is a complex process which consists of membrane depolarization, increases in cytosolic Ca^{2+} and ATP-dependent cross-bridge cycling. Each of these events are known to initiate intracellular signaling cascades. A major purpose of my thesis was to determine which of these events are responsible for contractile activity-induced alterations in the expression of nuclear genes encoding mitochondrial proteins. This was accomplished by systematically disrupting muscle contraction at various levels and examining the consequences on gene expression. One of the inhibitors used, 2,3-butanedione monoxime (BDM), disrupts muscle contraction by inhibiting myosin ATPase activity (Higuchi and Takemori, 1989) and preventing ATP-dependent cross-bridge cycling (Yagi et al., 1992). In addition, BDM is known to inhibit muscle contraction with no detrimental effects on Ca^{2+} transients at concentrations that are greater than those used in my study (1.5 mM; Eble et al., 1998; Yagi et al., 1992). BDM has also been shown to affect diatom gliding in microalgae (Poulson et al., 1999) and oligodendrocyte migration in response to PDGF. These effects are elicited by the actions of BDM on myosin contained within the cytoskeleton. The cytoskeleton is capable of inducing alterations in intracellular signaling (Simpson and Armstrong, 1999), and this may be a potential drawback to the use of BDM in my thesis. However, BDM effects on cytoskeletal myosin ATPase activity and

cell spreading occur at concentrations well above those used in our study (10 mM; Cramer and Mitchison, 1995). Thus, the concentration of BDM used to disrupt muscle contraction in Manuscript 3 was chosen in order to minimize the involvement of these potential side-effects of BDM. As such, I feel that the use of this drug to prevent muscle contraction at the level of cross-bridge formation is justified. However, I do acknowledge that other potential effects of BDM exist and have attempted to experimentally minimize their confounding influence on the data collected.

The introduction of plasmid DNA into mammalian cells is commonly used to study the contribution of a given gene, and its protein product, to the regulation of phenotype expression. DNA is often transiently introduced into cells in culture using a calcium phosphate method. However, this method can prove tricky and may not be suitable for use in all cell types. The use of poly-L-ornithine and DMSO shock is a much simpler method and has been used in muscle cell cultures (Freyssenet et al., 1999; Robey et al., 1996). A 5-10 % transfection efficiency has been reported for this method, with similar results obtained using the calcium phosphate technique (Robey et al., 1996). This value is below what is routinely observed by others using calcium phosphate, and this may reflect variability according to cell type and laboratory preparations. Transfection efficiency was not measured in the current study, since this protocol has been used by others (Freyssenet et al., 1999; Robey et al., 1996) and we were confident that the reporter genes of interest were being expressed to adequate levels. There was no apparent reason to assume that large variations in transfection efficiencies existed for stimulated or control cells. The cells were untreated

and identical to each other at the time of transfection, and control and stimulated cells were transfected at the same time. If the overall transfection efficiency was increased (e.g. 80 %), the relative rates between stimulated and control cells should persist, although the absolute expression of the promoter would be expected to be equally higher in both groups of cells. Therefore, although the efficiency of transfection is recognized to be far below 100 %, we are confident that the relative values that we observed are valid, and introducing more DNA would not alter our interpretation.

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APPENDIX 1

Extended Methods

Method 1 ***In vitro*** **mRNA DECAY**

Rationale: Many of the mRNAs which encode skeletal muscle proteins are very stable, making investigations of mRNA turnover *in vivo* difficult. The use of an in vitro system addresses this problem by allowing the creation of mRNA degradation conditions in which mRNA decay can proceed at a rapid rate.

Preparation of Cytosolic Extracts

1. In a 13 ml Sarstedt tube homogenize skeletal muscle powders (50 - 100 mg) 3 x 10 sec (10 mm probe; 70% maximum) in 1 ml of homogenisation buffer.
2. Centrifuge the homogenates for 15 min at 5,000g (4°C). Transfer the supernate to a 13 ml Sarstedt tube.
3. Centrifuge the supernate at 15,000g (4°C) for 15 minutes and transfer the resulting post-mitochondrial supernate (S15) to a sterile eppendorf tube.
4. Determine the protein concentrations of the S15 fractions using the Bradford total protein assay.

In vitro **Decay Reaction**

1. Incubate total RNA (30 μg) and S15 extract (45 μg) in a sterile eppendorf tube. Volume the reaction up to 300 μl with sterile DEPC treated water and incubate at 37°C.
2. Remove aliquots (100 μl) after the desired time periods and transfer them to a sterile eppendorf tube containing 100 μl of phenol. Mix vigorously.
3. Spin in a microcentrifuge for 30 seconds.

4. Transfer the aqueous phase to a sterile eppendorf tube. Add 100 µl of phenol/chloroform/isoamyl alcohol (25:24:1) and mix vigorously.
5. Spin in a microcentrifuge for 30 seconds.
6. Transfer the aqueous phase to a sterile eppendorf tube. Add 100 µl of chloroform/isoamyl alcohol (24:1) and mix vigorously.
7. Spin in a microcentrifuge for 30 seconds.
8. Transfer the aqueous phase to a sterile eppendorf tube. Add 10 µl of 3 M Na Acetate (pH 5.2) and 300 µl of 100% ethanol. Mix vigorously and then precipitate at -70°C for 1 hour or at -20°C overnight.
9. Spin in a microcentrifuge for 15 minutes at 4°C.
10. Wash the pellet with 75% ethanol and spin in a microcentrifuge for 5 minutes at 4°C. Pour off the supernate.
11. Cover the eppendorf with parafilm and poke holes in the covering. Place samples in the vacuum dessicator for 5 minutes (maximum) being careful not to "over dry" the pellet.
12. Resuspend the pellet in 10 µl of sterile DEPC H_20. Add 3 µl of 0.5 mg/ml EtBr and 12 µl of RNA sample buffer to the RNA and run on a 1% agarose gel.
13. Transfer the RNA to a nylon membrane (Hybond-N) overnight and fix the RNA to the membrane by exposing the blot to ultraviolet light on the transilluminator for 10 minutes (RNA side down).

SOLUTIONS

Homogenization Solution	**For 10 ml**
25 % glycerol	2.5 ml of sterile 100% stock solution
0.42 M NaCl	1 ml of a 4.2 M stock solution
1.5 mM $MgCl_2$	100 µl of a 150 mM stock solution
0.2 mM EDTA	4 µl of a 0.5 M stock solution
20 mM HEPES (pH 7.9)	500 µl of a 400 mM stock solution
0.5 mM DTT	10 µl of a 0.5 M stock solution
0.5 mM PMSF	10 µl of a 0.5 M stock solution
DEPC water	5.876 ml

Method 2 Gene Injection into rat tibialis anterior muscle

References

Wolff et al. Science 247: 1465-1468, 1990

Wolff et al. BioTechniques 11: 474-485, 1991

Davis et al. Human Gene Terapy 4:151-159, 1993

Materials

1 cc syringe without needle

27 gauge needle (PrecisionGlide, Becton Dickinson, NJ, USA) through Fischer forceps (sterile)

scissors (sterile)

5-0 silk braided (Ethicon, inc.Peterborough, Canada)

Plasmid DNA: 50 μg of plasmid of interest + 50 μg of plasmid used to the assess injection efficiency in 100 μl of normal sterile saline solution (use DPA assay to measure DNA concentration). The concentration must be above 1 μg/μl.

Procedure

1. Anaesthetize the animal with an intraperitoneal injection of sodium pentobarbital (40 mg/kg).
2. Shave the lower hindlimb of the animal is shaved.

3. Sterilize the incision by applying iodine to the shaved area (Proviodine solution, Rougier Inc. Chambly, Que)
4. Place animal on right side allowing clear access to the injection site of the left hindlimb.
5. Make a small incision in the skin (0.4 cm) so that the underlying muscle can be directly visualized. Do the incision at the upper part of the TA.
6. Fill the syringe up with the DNA solution (100 µl total) from 0.1 to 0.2 cc (see the graduations on the syringe). Make sure that the needle is tightly fixed on the syringe.
7. Perform the injection (30 sec) by maintaining a very small angle between the muscle and the syringe. Do not go deeper than 1 to 3 mm. Go from 0.2 to 0.1 cc on the syringe (avoid injecting air).
8. Remove the needle very gently from the muscle (10 sec). If the injection has been properly done, you can not see any leak from the muscle. Wait 5 minutes.
9. Suture the skin with the 5-O silk braided. One knot should be enough.
10. Allow the animal to recover without any further handling. Do not massage or put any pressure on the injected muscle. This has been shown to markedly reduce gene expression of the reporter gene (Davis et al. Human Gene Therapy 4:151-159, 1993).

Method 3 Chloramphenicol acetyltransferase (CAT) assay

1. Reaction mix:

Reagent	**(µl)**	**Notes**
0.25 M Tris HCl (pH 7.9)	18.9	Variable to a total of 28.9 μl
Extract*	10.0	
0.1 mM ^{14}C-chloramphenicol**	9.1	Depends on initial concentration
10mM Acetyl CoA***	2.0	Stored at -20°C, don't reuse
TOTAL	40.0	

*Amount of extract depends on the strength of the promotor and transfection efficiency.

** D-threo-[dichloroacetyl-1-^{14}C]-chloramphenicol (code CFA.754), 50 μCi, MW = 325, SA = 56 mCi/mmol, 172 μCi/mg, concentration = 25 μCi/ml, final chemical concentration = 0.1 mM, 0.23 μCi/reaction.

*** 10 mM Acetyl CoA, lithium salt = 8.8 mg/ml H_2O.

2. Incubate assay tubes at 37°C for 30 to 120 minutes, depending on the amount of activity expected. If ≥ 1 hour is expected, double the [Acetyl CoA] to a final concentration 1 mM to prevent substrate limitation. The assay can be repeated with stored extract at higher volume, with more Acetyl CoA, for longer time, etc. to get detectable results. During the reaction time, prepare the TLC chamber.
3. Extract chloramphenicol by adding 0.5 ml ethyl acetate; cap tightly, vortex vigorously and spin for 30 seconds in the microfuge.
4. With Pasteur pipette, transfer organic (upper) phase to disposable eppendorf tube.

5. Evaporate the ethyl acetate under vacuum in a dessicator to dryness (takes 20-30 minutes).
6. Resuspend the invisible residue by adding 25 µl of ethyl acetate.
7. Application of the Samples

   Tape the TLC plate on a glass plate with the even edge near the syringes.

   Take up half of the sample volume (7.5 µl) and spot on the pre-marked TLC plate

   Allow the spot to dry and apply the remaining 7.5 µl of the sample.

8. Preparation of the TLC Chamber

   Prepare 95:5 chloroform:methanol (v/v) = 142.5 ml Chloroform : 7.5 ml MeOH.

   Add to the chamber and cover using a glass plate sealed with grease and weighed down with lead. Do this 30-60 minutes before the TLC run. (This prepares the atmosphere - particularly if filter paper surrounds the back and sides of the chamber to absorb vapours).
9. TLC Run

   Clamp the sides of the TLC plate to the glass with small clips - <u>LABEL IT</u> with a pencil, top left.

   Put it in a far side of chamber, tilted away to the back side, so that the solvent front is visible.

   Allow 30-45 minutes for the solvent to reach approximately 2 cm from the top of the TLC plate. (Best results are obtained with <u>fresh</u> solvent + <u>60 minutes</u> of pre-incubation

of chamber).

10. Allow the plate to air dry for 3 minutes, remove the tape and glass and cover with Saran wrap.
11. Place on Instantimager until a reliable signal is visible. This time will vary depending on the signal strength (i.e. -726 bp cytochrome c promoter: 1 hour; -66 bp cytochrome c promoter: overnight).
12. Quantify blots and express acetylated forms (upper 2 bands) as a percentage of the total (all 3 bands). This gives CAT activity as a percent conversion.

Method 4 Electrical Stimulation of Myocytes in Culture

Cells: C2C12 murine myocytes

Dishes: 100 x 20 mm plastic non-coated culture dishes (Sarstedt 83.1802) are coated with 1 ml of 0.2% gelatin. The plates are then left in the hood overnight (lids off) under the germicidal (U.V.) light.

Modifications:

1. Punch 2 holes (7 cm apart) in the lids of the culture dishes, at the mid-line of the lid, with a heated syringe needle (18 gauge).
2. Permanently solder a 9.4 cm piece of platinum wire to a #8 hexagonal nut. Place the platinum wire through the holes in the lid (from above) and fix the hexagonal nut to the plastic using water tight silicone.
3. Bend the platinum wire such that it runs close to the bottom of the culture dish (not touching) and it runs at an ≈45° angle from the other hole in the lid. Bend the platinum wire back towards the lid of the dish 5 cm from the first bend. Fix the end of the wire to the underside of the dish with silicone.
4. Screw the connector for the banana plug into the hexagonal nut. It is possible to make the connection more permanent by CAREFULLY soldering the connector to the hexagonal nut (watch for heat build-up, the lids may melt).
5. Repeat steps 2 -4 for the other hole in the lid. This preparation should result in a final configuration where there are 5 cm pieces of platinum wire running parallel to each other on each culture dish lid (Fig. 1).

6. To sterilize the lids, expose them to the germicidal (U.V.) light overnight in the culture hood before they are used. During the stimulation protocol, wiping the electrodes with a kimwipe and 70% ethanol will suffice to maintain sterile conditions.

Medium: Cells are allowed to proliferate in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, D-5796) supplemented with 10% fetal bovine serum (Summit Biotechnologies, FP-100-05) and 1% penicillin/streptomycin (Sigma, P-0781) until myoblasts reach 90-100% confluence (3 days). Differentiation is then induced by switching the cells to DMEM supplemented with 5% heat inactivated horse serum (Life technologies, 16050-114) and 1% penicillin/streptomycin. Stimulation of the cells is routinely carried out when the myotubes are 90-100% confluent (8 days).

Stimulator: The stimulator was manufactured by the York University Electronics Shop with the following specifications:

A) Gange bipolar output (i.e., plus and minus amplitude adjustable using one knob).

B) Output voltage range is 0 to +300V and -300V.

C) Output current is 1A max.

D) Output pulse duration is adjustable from 0.001 to 0.1 seconds (10-1 kHz).

E) Output pulse repetition rate is adjustable from 0.0005 to 0.01 seconds (100-2 kHz).

F) Polarity duration range is adjustable from 1 to 100 seconds (0.01 to 1 Hz).

G) Polarity duration range defines the time duration for the output "pulse burst" to be positive before switching to a similar negative (amplitude) pulse burst (i.e. positive and negative duration are of equal value except for the amplitude).

H) Culture dishes were modified with Johnson miniature banana jacks (Electrosonic, E108-1021-001).

I) 20 (AWG) gauge wire was used with Johnson miniature banana plugs (Electrosonic, E108-1003-001) soldered at each end to facilitate connection to the dishes.

CO_2 incubator: Sanyo (MCO-17A) with a rear port to allow access of the electrodes to the incubator from the stimulator unit.

Typical use: Myotubes can be routinely stimulated in a PARALLEL circuit using voltages as low as 40 V but we typically use 65 V (1.2 V/cm^2). Cells are stimulated at a frequency of 5 Hz (4 ms duration) for durations of a few minutes up to a few days. This experimental set up results in observable synchronous contractions. Cells can be stimulated for up to 8 hrs/day for up to 4 days, although we typically stimulate for 3 hrs/day. Attempts to stimulate cell for 24 hrs/day have been unsuccessful to date, possibly due to the higher strength of contraction of the large myotubes on the 100 mm dishes compared to those on 35 mm dishes. We see little cell detachment after up to 4 days of stimulation (3 hrs/day) and it is likely that cells can be treated for longer periods (>4 days).

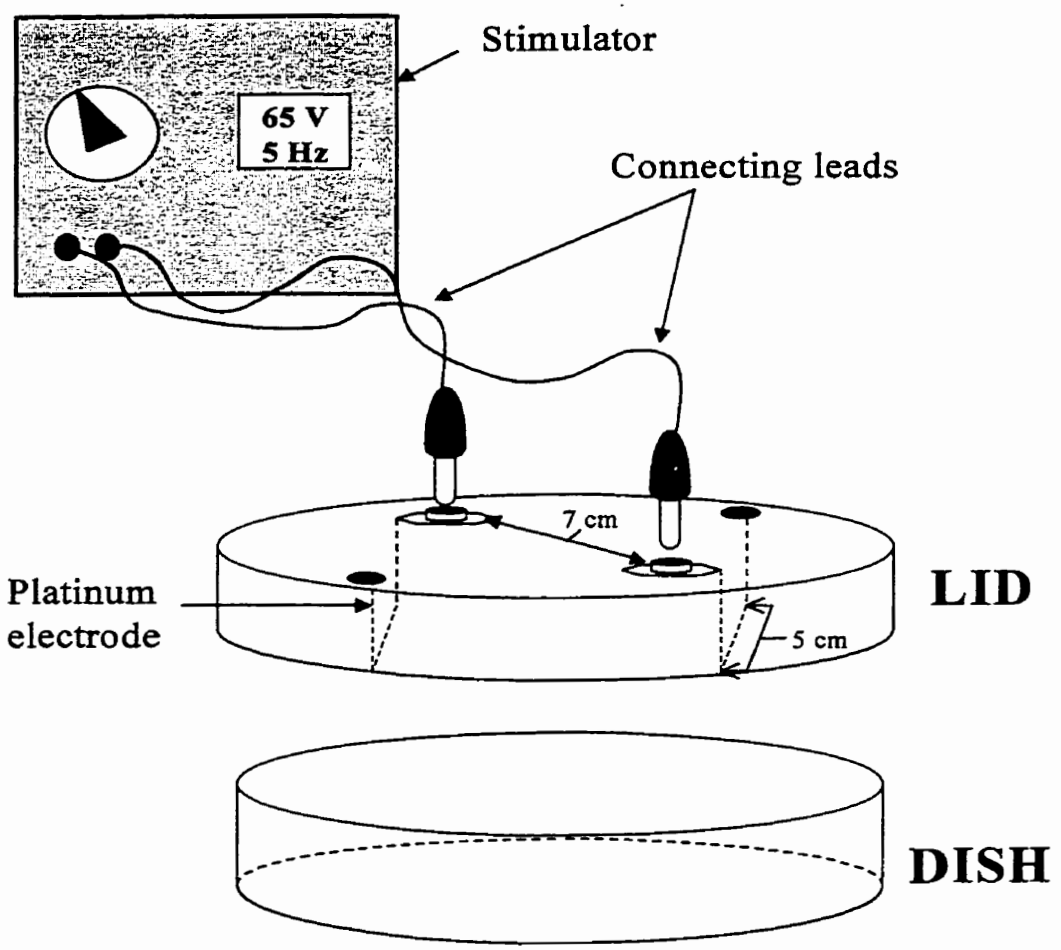

Figure 1. Schematic of the apparatus for electrical stimulation of C12C12 skeletal muscle cells in culture.

APPENDIX 2

OTHER CONTRIBUTIONS

I have also made contributions to the manuscripts listed below over the duration of my Ph.D.

1. Grey, J.Y., **M.K. Connor** and D.A. Hood. Tom20-mediated protein import in muscle cells during differentiation. Effect of thyroid hormone, forced overexpression and inhibition using anti-sense oligonucleotides. Submitted to *Biochem. J.* Sept. 1999.

2. **Connor, M.K.**, O. Bezborodova and D.A. Hood. Effect of contractile activity on protein turnover in skeletal muscle mitochondrial subfractions. *J. Appl. Physiol.* (in press, 2000).

3. Escobar, C.P., **M.K. Connor**, J.W. Gordon, P. Adhihetty and D.A. Hood. Fiber-type specific response of HSP70 to contractile activity. To be submitted to *J. Appl. Physiol.*

4. Hood, D.A, A. Balaban, **M.K. Connor**, E.E. Craig, M.L. Nishio, M. Rezvani and M. Takahashi. Mitochondrial biogenesis in skeletal muscle. *Can. J. Appl. Physiol.* 19: 12-48, 1994.