Title
Effects of microgravity on myogenic factor expressions during postnatal development of rat skeletal muscle

Journal
Journal of Applied Physiology, 92(5)

ISSN
8750-7587 1522-1601

Authors
Inobe, Manabu
Inobe, Ikuko
Adams, Gregory R
et al.

Publication Date
2002-05-01

DOI
10.1152/japplphysiol.00742.2001

J Appl Physiol 92: 1936–1942, 2002.
First published January 18, 2002; 10.1152/japplphysiol.00742.2001.

Effects of microgravity on myogenic factor expressions during postnatal development of rat skeletal muscle

MANABU INOBE,[1,2] IKUKO INOBE,[1] GREGORY R. ADAMS,[3] KENNETH M. BALDWIN,[3]
AND SHIN'ICHI TAKEDA[1]
[1]*Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8502;* [2]*Department of Space Experiment, National Space Development Agency of Japan, Tsukuba, Ibaraki 305-8505, Japan; and* [3]*Department of Physiology and Biophysics, University of California, Irvine, California 92697*

Received 20 July 2001; accepted in final form 10 January 2002

Inobe, Manabu, Ikuko Inobe, Gregory R. Adams, Kenneth M. Baldwin, and Shin'ichi Takeda. Effects of microgravity on myogenic factor expressions during postnatal development of rat skeletal muscle. *J Appl Physiol* 92: 1936–1942, 2002. First published January 18, 2002; 10.1152/japplphysiol.00742.2001.—To clarify the role of gravity in the postnatal development of skeletal muscle, we exposed neonatal rats at 7 days of age to microgravity. After 16 days of spaceflight, tibialis anterior, plantaris, medial gastrocnemius, and soleus muscles were removed from the hindlimb musculature and examined for the expression of MyoD-family transcription factors such as MyoD, myogenin, and MRF4. For this purpose, we established a unique semiquantitative method, based on RT-PCR, using specific primers tagged with infrared fluorescence. The relative expression of MyoD in the tibialis anterior and plantaris muscles and that of myogenin in the plantaris and soleus muscles were significantly reduced ($P < 0.001$) in the flight animals. In contrast, MRF4 expression was not changed in any muscle. These results suggest that MyoD and myogenin, but not MRF4, are sensitive to gravity-related stimuli in some skeletal muscles during postnatal development.

spaceflight; MyoD-family transcription factor; semiquantitative reverse transcriptase-polymerase chain reaction; hindlimb muscle

ADULT SKELETAL MUSCLES ARE COMPOSED of various types of fibers, each of which displays unique biochemical and contractile properties. These differences are partly due to the pattern of myosin heavy chain (MHC) isoforms that is expressed in each fiber type. At least four isoforms of adult MHC, designated slow (type I), fast IIa, fast IIx, and fast IIb, have been identified (9, 30). At birth, rodent skeletal muscles do not express appreciable levels of any of the adult forms of MHC. Thus rodent skeletal muscles develop postnatally from embryonic and neonatal to adult phenotypes (29). Postnatal development of rodent skeletal muscle is well defined (4, 13, 27, 29, 33), and this process occurs within 1 mo after birth (4). In other words, this time frame represents a critical window in which the skeletal muscles undergo a rapid muscle mass growth and MHC phenotype differentiation (4). It is apparent that neural and humoral factors regulate MHC transition from embryonic/neonatal to adult isoforms (29). Normal innervation appears to be needed for development of the slow (type I) isoform, whereas formation of fast (type II) isoforms, especially the IIb isoform, is dependent on the level of serum thyroid hormone (29). Thus both factors cooperate to differentiate each skeletal muscle into its respective inherent phenotype.

It has been reported that short-term spaceflight causes adult skeletal muscles to atrophy (10, 11, 14, 18, 25, 28), and a transition of the fiber type from the slow (type I) to the fast (type II) phenotype has been also observed (6, 11, 12, 14, 15, 25, 36). Therefore, weight bearing also affects the properties of each skeletal muscle.

The effects of gravity on the composition of adult skeletal muscle fiber have been well investigated by using rats flown in space, but the role of gravity in the postnatal development of skeletal muscle has not been addressed sufficiently. Recently, Adams et al. (2) showed that exposure of euthyroid neonatal rats to microgravity resulted in repression of type I MHC gene expression in the soleus (So) muscle, whereas type IIa and type IIx MHC proteins increased markedly. In contrast, slight modulation of the MHC profile was detected in fast-twitch muscles such as the plantaris (Pl). This involved an increase of type IIb MHC protein and a concomitant decrease of type IIa and type IIx MHC proteins (2). Thus exposure of neonatal rats to microgravity induces dramatic changes in muscle MHC phenotype in both fast- and slow-twitch muscles. In addition, Adams et al. (2) also provided evidence that the modification of MHC gene expression was regulated at the level of transcription/pretranslation. However, the regulatory mechanisms controlling MHC gene expression during postnatal development by load-bearing activity remain to be elucidated.

Four myogenic helix-loop-helix transcription factors (MyoD, myogenin, Myf-5, and MRF4) are expressed

---

Address for reprint requests and other correspondence: S. Takeda, Dept. of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan (E-mail: takeda@ncnp.go.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

specifically in skeletal muscle and are known to regulate its development/maturation process (8, 31, 35). These transcription factors have been shown to bind to specific DNA motifs, e.g., E-box sequences, resulting in activation of muscle-specific genes. MyoD-family transcription factors may be candidate regulators that mediate the effects of extrinsic factors during development. In fact, MyoD-family molecules are expressed in different patterns depending on the muscle phenotype (17, 19, 32). MyoD and myogenin mRNAs accumulate selectively in adult muscles according to their contractile properties. MyoD is prevalent in fast-twitch (type II) muscle fibers, whereas myogenin is preferentially expressed in slow-twitch (type I) muscle fibers (17, 19, 32).

In this study, we examined the relative expression of three MyoD-family transcription factors (MyoD, myogenin, and MRF4) in neonatal rat hindlimb muscles after 16 days of spaceflight to address the role of gravity on muscle development. Tissue samples analyzed in this study were obtained from rats that had been exposed to microgravity in the Neurolab Mission (STS-90) and demonstrated that normal MHC transition in various skeletal muscles was prevented (2, 3). Although it is difficult to quantify the relative expression of MyoD-family transcription factors due to their low expression and the lack of sufficient amounts of muscle tissues in neonatal animals, a RT-PCR methodology was developed to address these issues because RT-PCR is highly sensitive in detecting mRNA isolated from small amounts of sample. However, as initially used, RT-PCR lacked sufficient accuracy in quantitation. Several modifications to overcome this defect have been tested and reported (19, 34). In the present paper, we report a unique new method, based on RT-PCR, using a PCR primer labeled with infrared fluorescence, thereby making it possible to quantify the relative expression of three myogenic factors accurately and conveniently. The relative expression of MyoD was significantly reduced ($P < 0.001$) in the tibialis anterior (TA) and Pl muscles of flight animals, and that of myogenin was again significantly reduced ($P < 0.001$) in the Pl and So muscles of the flight group. In contrast, MRF4 expression was not changed in any muscle. Taken together, these results indicate that the differentiation/maturation process of skeletal muscles is likely influenced by gravity, possibly through expression of key myogenic factors.

MATERIALS AND METHODS

Experimental groups and tissue processing. Seven-day-old Sprague-Dawley rats were used in a National Aeronautics and Space Administration (NASA) project designated Neurolab. These animals were randomly divided into three groups: *1*) a flight ($n = 6$) group, *2*) a vivarium ($n = 8$) group, and *3*) an asynchronous ground control (AGC; $n = 8$) group. Flight group animals flew aboard the shuttle housed in a rodent animal holding facility cage along with their nursing mother. These cages were located in the cargo bay of the Shuttle Transport System-90. Vivarium and AGC groups, which were maintained in a rodent animal holding facility cage prototype and in a conventional cage on the ground, respectively, were used as control groups. The space shuttle was launched from the Kennedy Space Center on April 17, 1998, and landed on May 3, 1998. The TA, Pl, So, and medial gastrocnemius (MG) muscles were removed from the hindlimb musculature of the flight animals as described in detail previously (3). Flight animals used in this study were the same animals employed by Adams and colleagues (2, 3). The mid portion of each muscle was transected, removed, and placed on a cork by using tragacanth gum (Wako Pure Chemicals, Osaka, Japan). The tissues were then rapidly frozen by using isopentane cooled by liquid nitrogen. These tissue samples were subjected to mRNA analysis. Muscles from the control groups were isolated and analyzed in an identical manner. All experimental procedures were approved by the NASA Institutional Animal Care and Use Committee.

Analysis of expression of three myogenic factors by semiquantitative RT-PCR. Total RNA was isolated by using RNAsol B (TEL-TEST, Friendswood, TX) from 15-μm muscle sections. For this purpose, 10 sections of each TA and MG muscle and 15 sections of each Pl and So muscle were used. cDNA was then prepared from total RNA samples by using random hexamer primers included in a first-strand cDNA synthesis kit (Pharmacia, Uppsala, Sweden). These cDNAs were used as templates of the PCR reactions described below.

To amplify DNA fragments derived from MyoD, myogenin, MRF4, and G3PDH, we prepared specific primer pairs, which were located in the coding region and included at least one intron between each pair. The sequences of these primers are presented in Table 1. The reverse primer was labeled with IRD-41 at the 5′ end to detect infrared fluorescence emitted from the PCR product. PCR reaction was performed by using LA-*Taq* DNA polymerase (Takara, Kyoto, Japan) in a volume of 20 μl, which included 1 μl of cDNA solution. Amplification was carried out by repeating the following cycles: 94°C for 1 min, 66°C for 1 min, and 72°C for 1 min. For suitable detection of the PCR product, the cycle number was optimized according to the amount of cDNA; however, usually 27 cycles were performed for MyoD, 24 cycles for myogenin and MRF4, and 15 cycles for glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Aliquots were loaded on 7% denatured polyacrylamide gel containing 7 M urea and analyzed by a DNA sequencer 4200 (LI-COR Biosciences, Lincoln, NE).

To correct for amplification efficiency, which may differ among the reactions, an external control DNA fragment was used as a competitor. An identical amount of competitor was added to both the test samples and standard reaction mixtures to avoid variation among samples. The amount of competitor DNA was optimized around 5×10^{-22} mol for MyoD, 5×10^{-21} mol for myogenin, 1×10^{-19} mol for MRF4, and 1×10^{-17} mol for G3PDH per tube. To obtain actual expression, the density of the band corresponding to the target cDNA was divided by that corresponding to the competitor. Both densities were calculated by using National Institutes of Health Image software (version 1.61) from the digital data collected by the DNA sequencer 4200. To estimate the number of cDNA molecules, standard DNAs, serially diluted, were assayed as well as cDNA samples to produce a standard curve for every experiment. In this assay, identical efficiency of the reverse transcription reaction for each of the mRNAs examined was assumed.

Construction of the standard and the competitor DNA. To prepare the standard DNA, PCR products amplified under the conditions described above were used, except that a nonlabeled reverse primer was used. These standards were cloned into a pCRII vector (Invitrogen, Carlsbad, CA). After

Table 1. *Oligonucleotide primers used for RT-PCR and fragments obtained by digestion of the products*

Primer Set	Position	Length, nt	Diagnostic Restriction Enzyme	Restriction Fragment, nt
MyoD				
5′-CAACTGCTCTGATGGCATGATGG	819–841	267	Eagl	147
3′-CTGTAGGAGTTCGCTACGTCTTGT-IRD41	1062–1085		BstXI	117
Myogenin				
5′-GCAGTGCCATCCAGTACATTGAGC	402–425	285	Pvull	84
3′-CACCTCCTATACAGACAGTGGAAGG-IRD41	661–685		Pstl	148
MRF4				
5′-TCAACTACATTGAGCGTCTGCAGG	457–480	273	BstXI	101
3′-GGTAGCACCTGTCATAAAGGAGTC-IRD41	706–729		Pstl	139
G3PDH				
5′-TCTTCACCACCATGGAGAAGGCTG	367–390	262	Mscl	82
3′-GTAGTGACGGTGAGTCTTCTGACA-IRD41	605–628		EcoT22I	117

Primer sets are used as an infrared fluorescence tag; IRD41 was coupled with the reverse, but not the forward, primer at 5′ termini. Positions are indicated by numbers corresponding to the site of mRNA sequence. mRNA sequences are available in the GenBank. Accession numbers are as follows: MyoD, M84176; myogenin, M24393; MRF4, M27151; glyceraldehyde-3-phosphate dehydrogenase (G3PDH), X02231.

verification of the insert by sequencing, PCR products were reamplified from these plasmids by using the same primers and then loaded on 2% agarose gels in the presence of 0.5 μg/ml EtBr. PCR products were purified from gels by using a QIAquick gel extraction kit (QIAGEN, Chatsworth, CA), and DNA concentration was determined on the basis of the absorbance at 260 nm. Competitor DNA fragments were constructed by oligonucleotide overlap extension and amplification by PCR (16). The final product had a 26-bp DNA insertion derived from the multicloning site of pBluescript II (GCTTATCGATACCGTCGACCTCGAGG). Competitor DNA fragments were prepared for each specific standard DNA product prepared above.

Restriction enzyme analysis. To confirm exclusive amplification of products derived from MyoD, myogenin, MRF4, and G3PDH mRNA under the RT-PCR conditions used in this study, PCR products were subjected to a series of restriction digestions by using several restriction endonucleases (see Table 1). Product identity was confirmed by obtaining the correct size of restriction fragments on the basis of published mRNA sequence for each of the studied mRNAs (see Table 1 for expected fragment sizes).

Statistical analysis. All statistical tests were made by using an independent t-test. Statistical tests were considered significant at $P < 0.001$ or $P < 0.005$.

RESULTS

Establishment of unique semiquantitative RT-PCR for MyoD, myogenin, MRF4, and G3PDH. To analyze the expression of the MyoD family of transcription factors, which are expressed at a very low level in skeletal muscle samples available only in small amounts because the muscles do not grow appreciably during spaceflight (3), we needed to establish a highly sensitive and semiquantitative method based on RT-PCR. Therefore, we applied an infrared fluorescence-tagged primer to develop a unique RT-PCR method as described in MATERIALS AND METHODS.

First, we confirmed exclusive amplification of products derived from MyoD, myogenin, MRF4, and G3PDH mRNA by restriction enzyme analysis (Fig. 1*A*). Treatment of RT-PCR products with two kinds of restriction enzymes resulted in their complete digestion, and the lengths of the fragments were consistent with our expectations. These results indicated that one can detect MyoD, myogenin, MRF4, and G3PDH mRNA separately in a muscle-specific manner (Table 2).

Due to variations in the PCR reaction, it was necessary to monitor the efficiency of amplification by means of an external standard. We prepared competitor DNAs, which were 26 bp longer than each PCR product, and included them in each of the reaction mixtures as the external control fragment. Figure 1*B* presents a representative result obtained from analysis of the serially diluted standard DNAs. We routinely obtained two major bands, as shown. When the density of the standard DNA band was divided by that of the corresponding competitor, a good linear relationship between amount of standard DNA and band density was obtained (Fig. 1*C*). Standard curves were generated for each specific cDNA and were used to estimate the number of MyoD, myogenin, MRF4, and G3PDH cDNA molecules in unknown samples.

Comparison of the expression of MyoD-family transcription factors in neonatal rat skeletal muscles. We investigated the expression of MyoD, myogenin, MRF4 and G3PDH in the control muscles. The mRNAs isolated from the TA, Pl, MG, and So muscles of the AGC rats were subjected to RT-PCR. The expression of MyoD, myogenin, and MRF4 genes was represented relatively as the number of cDNA molecules in a solution containing 1×10^5 G3PDH cDNAs under the presumption that no fluctuation of G3PDH expression was induced (22, 24) (Table 2). MRF4 expression was higher than that of myogenin, and myogenin expression was higher than MyoD expression in each of the different muscles. The significantly higher ratio of myogenin to MyoD expression in So muscle relative to the fast muscles examined (Table 2) indicated that this value was characteristic of So muscle's slow phenotype (17, 19, 32).

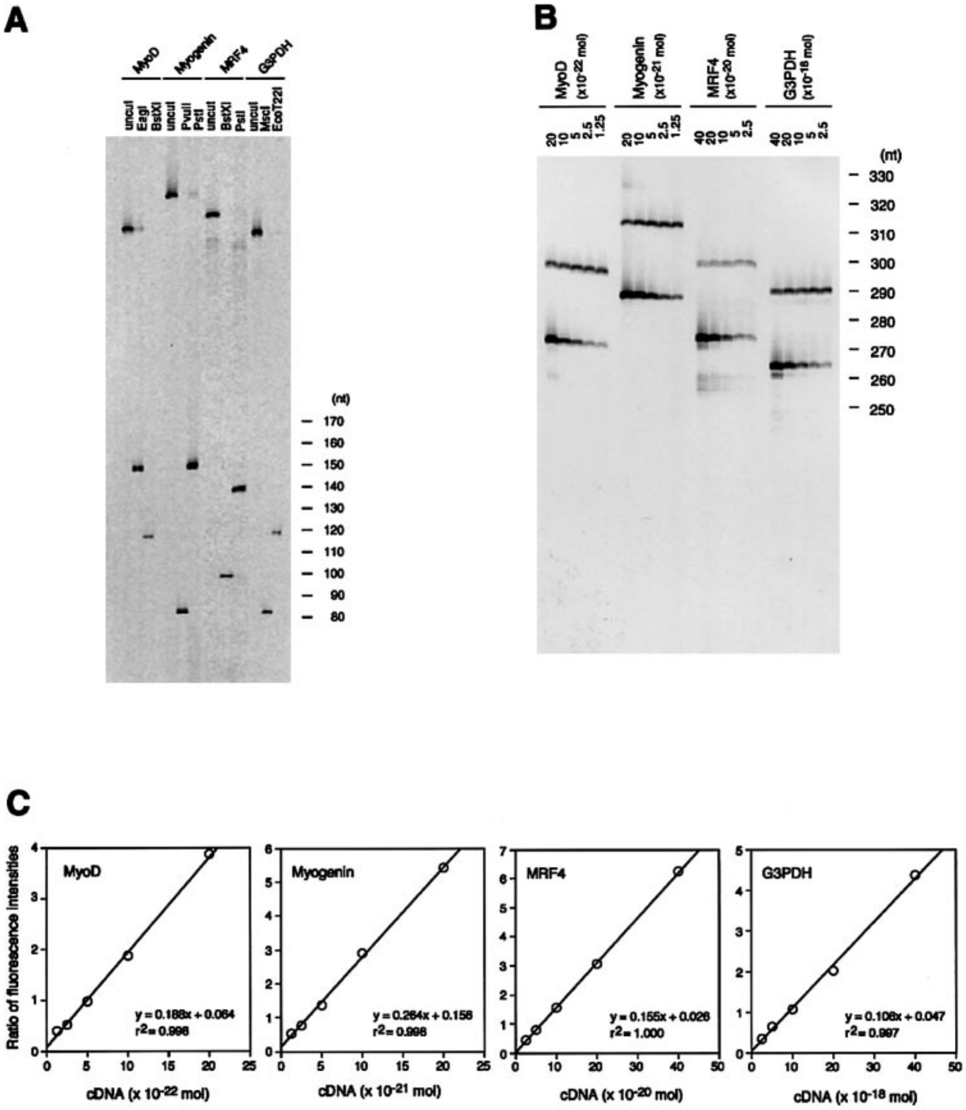

Fig. 1. Establishment of semiquantitative RT-PCR for MyoD, myogenin, MRF4, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). *A*: RT-PCR products were digested with the restriction enzymes indicated at *top*. Molecular size markers are indicated at *right*. Treatment of RT-PCR products with restriction enzymes generated shorter fragments of expected size on the basis of mRNA sequence information. *B*: various amounts of standard DNA, indicated at *top*, and competitor DNA were mixed in the tube and then coamplified by PCR. The amounts of competitor DNA were 5×10^{-22} mol for MyoD, 5×10^{-21} mol for myogenin, 1×10^{-19} mol for MRF4, and 1×10^{-17} mol for G3PDH. DNA molecular size markers are indicated at *right*. *C*: fluorescence intensities of the bands were determined by National Institutes of Health Image analysis software. Density of the standard DNA band was divided by its corresponding competitor DNA and plotted against the varied amounts of standard DNA. Data presented were derived from the experiment shown in Fig. 1*B*. Representative results were evaluated by linear regression analysis.

Effect of microgravity on the expression of MyoD-family transcription factors in neonatal rat skeletal muscles. MyoD, myogenin, and MRF4 expression in the TA, Pl, MG, and So muscles derived from flight, vivarium, and AGC rats was investigated (Fig. 2). MyoD expression was reduced significantly in the TA and Pl muscles in the flight group relative to the vivarium group. Relative expression of myogenin decreased significantly in the Pl and So muscles in the flight group compared with the vivarium group. In contrast, MRF4 expression was not changed in any muscle. These results indicated that exposure to microgravity resulted in downregulation of MyoD and myogenin gene expression in some neonatal skeletal muscles.

DISCUSSION

Previously, it was difficult to assess the exact expression of MyoD family transcription factors due to their low expression in skeletal muscle. Kraus and Pette (19) combined RT-PCR and ELISA methods to measure the mRNA of MyoD family transcription factors. This method was highly sensitive, but the procedure was very complicated (19). In this study, we established an

Table 2. *Expression of MyoD family transcription factors in various muscles of AGC rat*

Muscle	*n*	cDNA molecules/10^5 G3PDH cDNAs			Myogenin/MyoD
		MyoD	Myogenin	MRF4	
Tibialis anterior	8	7.96 ± 0.45	57.2 ± 2.68	949 ± 38.9	7.34 ± 0.52
Medial gastrocnemius	8	12.9 ± 0.72	80.1 ± 6.50	1060 ± 73.5	6.20 ± 0.32
Plantaris	6	13.5 ± 1.36	102 ± 11.6	1150 ± 240	8.10 ± 1.31
Soleus	7	8.29 ± 1.14	258 ± 14.7	1900 ± 98.8	34.1 ± 3.93*

Values of cDNA molecules of myogenic factors per 10^5 G3PDH cDNAs are means ± SE. Myogenin-to-MyoD ratios are also indicated. *$P <$ 0.005 vs. tibialis anterior, medial gastrocnemius, and plantaris muscles.

assay system based on RT-PCR that can quantify a low level of relative gene expression accurately and simply in samples weighing <1 mg. To establish this assay, we modified the RT-PCR method by using an oligonucleotide primer tagged with infrared fluorescence, making it possible to evaluate expression of several different genes by using a minimal amount of tissue sample. In fact, we could quantify the cDNA content of three myogenic transcription factors, MyoD, myogenin, and MRF4, in addition to a housekeeping gene, G3PDH, in the same sample. The three myogenic factors were expressed in order of MRF4 > myogenin > MyoD in all muscles analyzed from the hindlimbs of normal 23-day-old rats (Table 2). Moreover, the ratio of myogenin to MyoD expression was higher in the So muscle, which is dominated by the expression of slow-twitch fibers, compared with that seen in TA, MG, and Pl muscles. The latter muscles are biased toward expressing mainly fast-twitch fibers. These results are consistent with previous studies on adult rodents (17, 19, 32). In addition, it is obvious from restriction enzyme digestion that each fragment is amplified specifically during the PCR reaction, suggesting that our method is suitable for these analyses. In this study, expression of the housekeeping gene G3PDH was utilized as an internal standard due to accumulated confidence in its reliability (22, 24). However, we have to keep in mind reports (20, 21) that associate the dynamic regulation of G3PDH gene expression with age, myofiber phenotype, and lack of muscle loading. To increase reliability, several standard genes need to be incorporated into a method. In this regard, stable expression of the MRF4 gene observed after treatment in this study is consistent with previous reports (20, 24) regarding the stability of its expression in different conditions, indicating the RT-PCR method developed here was applied appropriately in this study.

Adams et al. (2) showed that exposure of euthyroid neonates to microgravity resulted in dramatic changes in muscle MHC phenotype in both fast- and slow-twitch muscles. In slow-twitch So muscle, a repression of type I MHC gene expression and an increase of type IIa and type IIx proteins were markedly induced. In contrast, a slight modulation, including an increase of type IIb MHC protein and a decrease of type IIa and type IIx MHC proteins, was detected in fast-twitch Pl

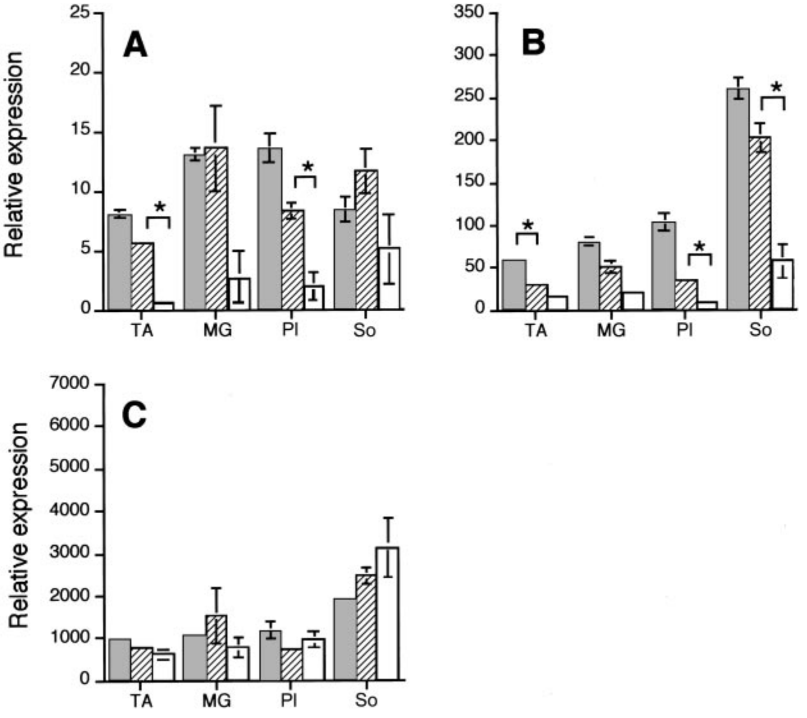

Fig. 2. Effect of microgravity on the expression of MyoD-family transcription factors in neonatal rat skeletal muscles. RNA isolated from flight (open bars), vivarium (hatched bars), and asynchronous ground control (shaded bars) rat skeletal muscles were analyzed by semiquantitative RT-PCR, described in MATERIALS AND METHODS. Expression of MyoD (*A*), myogenin (*B*), and MRF4 (*C*) is represented relative to the number of cDNA molecules of myogenic factors per 10^5 G3PDH cDNAs. Values are means ± SE. TA, tibialis anterior; MG, medial gastrocnemius; Pl, plantaris; So, soleus. *$P < 0.001$.

muscle. Thus, in the absence of normal weight-bearing activity, muscle phenotype becomes clearly biased toward a faster MHC profile. In view of the findings presented above, it was of interest to examine whether key myogenic factors in the developmental cascade are affected by this unique environment. In this study, neonatal rats exposed to spaceflight were analyzed for gene expression of the MyoD family transcription factors at 23 days of age after 16 days of spaceflight. Relative expression of MyoD in TA and Pl muscles and that of myogenin in Pl and So muscles of the flight animals were significantly reduced ($P < 0.001$) relative to AGC values (Fig. 2), whereas MRF4 expression was not changed. Taken together, these results indicate that the differentiation/maturation process of skeletal muscles is likely influenced by gravity, possibly through expression of key myogenic factors. The expression of myogenic factors in response to muscle unloading has been studied after hindlimb suspension in rats, which can mimic the conditions of spaceflight (7, 23). Mozdziak et al. (23) indicated that the expression of MyoD but not of myogenin increased in So muscle at mRNA levels after hindlimb suspension. In contrast, Alway et al. (7) showed that MyoD and myogenin mRNA levels were not altered in So muscle by hindlimb suspension, whereas these mRNAs dramatically increased in Pl muscle. This discrepancy in the modulation of myogenic factor expressions seems to be partly due to the age of the rats examined. It is reported that MyoD and myogenin expressions are closely correlated with age (7, 24). The high expressions of both mRNAs observed just after birth rapidly decreased and reached a minimum at ~4 mo after birth; then both increased and recovered in senescent rats >2 yr old (7, 24). Mozdziak et al. (23) and Alway et al. (7) examined the effect of muscle unloading in young adult rats aged 6 and 4 mo, respectively. Furthermore, the MHC phenotype differentiation occurred rapidly and was almost completed within 1 mo after birth (4). Taken together, the time frame examined in this study represents a critical period for analyses concerning the expression of three myogenic factors, MyoD, myogenin, and MRF4, that have been implicated in muscle development/differentiation processes (8, 31, 35). In the context of the above findings, Hughes et al. (17) suggested that MyoD and myogenin mediate both neural and humoral control of the postnatal development of skeletal muscle.

MRF4 expression was not influenced by short-term spaceflight. MRF4 is a myogenic factor expressed mainly after birth and is likely to have a role in the maintenance of skeletal muscles rather than development/differentiation processes, which may be controlled by MyoD, myogenin, and Myf-5 expression (26). MRF4, unlike MyoD and myogenin, sustains its steady expression independent of age. Furthermore, MRF4 expression is resistant to a lack of loading stimulation in adult skeletal muscles (7, 24). Tight regulation of MRF4 expression independent of the loading stimulus may again indicate a distinct role of MRF4 from MyoD and myogenin in the development of skeletal muscles.

In the context of the observations presented here, Adams and colleagues (1, 3) reported that blood and tissue levels of insulin-like growth factor (IGF)-I are tightly linked with muscle-loading activity. Somatic and muscle-specific IGF-I expression is impaired in developing euthyroid and hypothyroid neonates exposed to spaceflight (3). In addition, it recently has been reported that IGF-I induces the expression of MyoD-family transcription factors (5). Thus reduction of MyoD and myogenin expression in neonatal rats exposed to microgravity may be due to the reduction in IGF-I. Interestingly, thyroid hormone deficiency, in and of itself during neonatal development, results in lower levels of blood and skeletal muscle IGF-I expression (3, 4). This response essentially mimics that seen in euthyroid neonatal animals exposed to microgravity. However, the change in MHC profile in hypothyroid animals is totally different compared with that seen in euthyroid animals exposed to microgravity (2, 3). In the case of thyroid hormone deficiency in adult animals, MyoD gene expression, but not the myogenin gene, was suppressed (19). Thus different mechanisms may be involved in the regulation of postnatal development of rodent skeletal muscle compared with factors that alter gene expression and muscle homeostasis in the adult state.

In conclusion, given the fact that spaceflight induces slow to fast transitions in the MHC phenotype in both slow- and fast-twitch muscles of developing rodents (2) and given the fact that both MyoD and myogenin gene expression are repressed in both fast and slow muscles, such as the TA, Pl, and So muscles, under these same conditions, it seems reasonable to conclude that although these myogenic transcription factors are likely important during the growth and differentiation process of developing neonatal skeletal muscle, these factors are probably not the primary regulators controlling the MHC phenotype transitions seen in those neonatal muscles exposed to spaceflight. However, it is curious that in the So muscle of flight-exposed neonates, myogenin in particular was markedly reduced to levels in the range seen in the fast muscles of both flight- and ground-based neonates (Fig. 2). Because we have observed that both the proximal and distal regions of the type I MHC promoter contain numerous E-box elements, which are thought to interact with myogenic factors such as myogenin, marked repression of this factor in the neonatal So could act in consort with other regulatory transcription factors in modulating the downregulation of this gene in the So muscle of microgravity-exposed neonates. Clearly, more research is needed in further addressing the role of myogenic factors on muscle growth and differentiation processes.

We thank the members of the research support teams from the NASA Ames Research Center, John F. Kennedy Space Center, and National Space Development Agency of Japan. In paticular, we thank Gail Nakamura, Vera Vizir, Hisao Sato, and Masaru Uemura for experimental support.

Present address of M. Inobe: Division of Molecular Immunology, Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan.

REFERENCES

1. **Adams GR, Haddad F, and Baldwin KM.** Time course of changes in markers of myogenesis in overloaded rat skeletal muscles. *J Appl Physiol* 87: 1705–1712, 1999.
2. **Adams GR, Haddad F, McCue SA, Bodell PW, Zeng M, Qin L, Qin AX, and Baldwin KM.** Effects of spaceflight and thyroid deficiency on rat hindlimb development. II. Expression of MHC isoforms. *J Appl Physiol* 88: 904–916, 2000.
3. **Adams GR, McCue SA, Bodell PW, Zeng M, and Baldwin KM.** Effects of spaceflight and thyroid deficiency on hindlimb development. I. Muscle mass and IGF-I expression. *J Appl Physiol* 88: 894–903, 2000.
4. **Adams GR, McCue SA, Zeng M, and Baldwin KM.** Time course of myosin heavy chain transitions in neonatal rats: importance of innervation and thyroid state. *Am J Physiol Regulatory Integrative Comp Physiol* 276: R954–R961, 1999.
5. **Adi S, Cheng ZQ, Zhang PL, Wu NY, Mellon SH, and Rosenthal SM.** Opposing early inhibitory and late stimulatory effects of insulin-like growth factor-I on myogenin gene transcription. *J Cell Biochem* 78: 617–626, 2000.
6. **Allen DL, Yasui W, Tanaka T, Ohira Y, Nagaoka S, Sekiguchi C, Hinds WE, Roy RR, and Edgerton VR.** Myonuclear number and myosin heavy chain expression in rat soleus single muscle fibers after spaceflight. *J Appl Physiol* 81: 145–151, 1996.
7. **Alway SE, Lowe DA, and Chen KD.** The effects of age and hindlimb suspension on the levels of expression of the myogenic regulatory factors MyoD and myogenin in rat fast and slow skeletal muscles. *Exp Physiol* 86: 509–517, 2001.
8. **Arnold HH and Winter B.** Muscle differentiation: more complexity to the network of myogenic regulators. *Curr Opin Genet Dev* 8: 539–544, 1998.
9. **Baldwin KM.** Effects of altered loading states on muscle plasticity: what have we learned from rodents? *Med Sci Sports Exerc* 28: S101–S106, 1996.
10. **Booth FW and Criswell DS.** Molecular events underlying skeletal muscle atrophy and the development of effective countermeasures. *Int J Sports Med* 18: S265–S269, 1997.
11. **Caiozzo VJ, Baker MJ, Herrick RE, Tao M, and Baldwin KM.** Effect of spaceflight on skeletal muscle: mechanical properties and myosin isoform content of a slow muscle. *J Appl Physiol* 76: 1764–1773, 1994.
12. **Caiozzo VJ, Haddad F, Baker MJ, Herrick RE, Prietto N, and Baldwin KM.** Microgravity-induced transformations of myosin isoforms and contractile properties of skeletal muscle. *J Appl Physiol* 81: 123–132, 1996.
13. **D'Albis A, Couteaux R, Janmot C, and Roulet A.** Specific programs of myosin expression in the postnatal development of rat muscles. *Eur J Biochem* 183: 583–590, 1989.
14. **Desplanches D.** Structural and functional adaptations of skeletal muscle to weightlessness. *Int J Sports Med* 18: S259–S264, 1997.
15. **Haddad F, Herrick RE, Adams GR, and Baldwin KM.** Myosin heavy chain expression in rodent skeletal muscle: effects of exposure to zero gravity. *J Appl Physiol* 75: 2471–2477, 1993.
16. **Horton RM, Cai ZL, Ho SN, and Pease LR.** Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques* 8: 528–535, 1990.
17. **Hughes SM, Taylor JM, Tapscott SJ, Gurley CM, Carter WJ, and Peterson CA.** Selective accumulation of MyoD and myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. *Development* 118: 1137–1147, 1993.
18. **Jiang B, Ohira Y, Roy RR, Nguyen Q, Ilyina-Kakueva EI, Oganov V, and Edgerton VR.** Adaptation of fibers in fast-twitch muscles of rats to spaceflight and hindlimb suspension. *J Appl Physiol* 73: 58–65, 1992.
19. **Kraus B and Pette D.** Quantification of MyoD, myogenin, MRF4 and Id-1 by reverse-transcriptase polymerase chain reaction in rat muscles: effects of hypothyroidism and chronic low-frequency stimulation. *Eur J Biochem* 247: 98–106, 1997.
20. **Lowe DA, Degens H, Chen KD, and Alway SE.** Glyceraldehyde-3-phosphate dehydrogenase varies with age in glycolytic muscles of rats. *J Gerontol A Biol Sci Med Sci* 55: B160–B164, 2000.
21. **McCarthy JJ, Fox AM, Tsika GL, Gao L, and Tsika RW.** β-MHC transgene expression in suspended and mechanically overloaded/suspended soleus muscle of transgenic mice. *Am J Physiol Regulatory Integrative Comp Physiol* 272: R1552–R1561, 1997.
22. **Mozdziak PE, Greaser ML, and Schultz E.** Myogenin, MyoD, and myosin expression after pharmacologically and surgically induced hypertrophy. *J Appl Physiol* 84: 1359–1364, 1998.
23. **Mozdziak PE, Greaser ML, and Schultz E.** Myogenin, MyoD, and myosin heavy chain isoform expression following hindlimb suspension. *Aviat Space Environ Med* 70: 511–516, 1999.
24. **Musaro A, Cusella De Angelis MG, Germani A, Ciccarelli C, Molinaro M, and Zani BM.** Enhanced expression of myogenic regulatory genes in aging skeletal muscle. *Exp Cell Res* 221: 241–248, 1995.
25. **Ohira Y, Jiang B, Roy RR, Oganov V, Ilyina-Kakueva E, Marini JF, and Edgerton VR.** Rat soleus muscle fiber responses to 14 days of spaceflight and hindlimb suspension. *J Appl Physiol* 73: 51–57, 1992.
26. **Perry RL and Rudnicki MA.** Molecular mechanisms regulating myogenic determination and differentiation. *Front Biosci* 5: D750–D767, 2000.
27. **Picquet F, Stevens L, Butler-Browne GS, and Mounier Y.** Contractile properties and myosin heavy chain composition of newborn rat soleus muscles at different stages of postnatal development. *J Muscle Res Cell Motil* 18: 71–79, 1997.
28. **Riley DA, Ellis S, Slocum GR, Sedlak FR, Bain JL, Krippendorf BB, Lehman CT, Macias MY, Thompson JL, Vijayan K, and De Bruin JA.** In-flight and postflight changes in skeletal muscles of SLS-1 and SLS-2 spaceflown rats. *J Appl Physiol* 81: 133–144, 1996.
29. **Russell SD, Cambon NA, and Whalen RG.** Two types of neonatal-to-adult fast myosin heavy chain transitions in rat hindlimb muscle fibers. *Dev Biol* 157: 359–370, 1993.
30. **Schiaffino S and Reggiani C.** Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiol Rev* 76: 371–423, 1996.
31. **Smith TH, Block NE, Rhodes SJ, Konieczny SF, and Miller JB.** A unique pattern of expression of the four muscle regulatory factor proteins distinguishes somitic from embryonic, fetal and newborn mouse myogenic cells. *Development* 117: 1125–1133, 1993.
32. **Voytik SL, Przyborski M, Badylak SF, and Konieczny SF.** Differential expression of muscle regulatory factor genes in normal and denervated adult rat hindlimb muscles. *Dev Dyn* 198: 214–224, 1993.
33. **Whalen RG, Sell SM, Butler-Browne GS, Schwartz K, Bouveret P, and Pinset-Harstom I.** Three myosin heavy-chain isozymes appear sequentially in rat muscle development. *Nature* 292: 805–809, 1981.
34. **Wright C, Haddad F, Qin AX, and Baldwin KM.** Analysis of myosin heavy chain mRNA expression by RT-PCR. *J Appl Physiol* 83: 1389–1396, 1997.
35. **Yun K and Wold B.** Skeletal muscle determination and differentiation: story of a core regulatory network and its context. *Curr Opin Cell Biol* 8: 877–889, 1996.
36. **Zhou MY, Klitgaard H, Saltin B, Roy RR, Edgerton VR, and Gollnick PD.** Myosin heavy chain isoforms of human muscle after short-term spaceflight. *J Appl Physiol* 78: 1740–1744, 1995.