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Effects of spaceflight and thyroid deficiency on rat hindlimb development. II. Expression of MHC isoforms

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Adams, G. R., F. Haddad, S. A. McCue, P. W. Bodell, M. Zeng, L. Qin, A. X. Qin, and K. M. Baldwin. Effects of spaceflight and thyroid deficiency on rat hindlimb development. II. Expression of MHC isoforms. *J. Appl. Physiol.* 88: 904–916, 2000.—Both slow-twitch and fast-twitch muscles are undifferentiated after birth as to their contractile protein phenotype. Thus we examined the separate and combined effects of spaceflight (SF) and thyroid deficiency (TD) on myosin heavy chain (MHC) gene expression (protein and mRNA) in muscles of neonatal rats (7 and 14 days of age at launch) exposed to SF for 16 days. Spaceflight markedly reduced expression of the slow, type I MHC gene by ~55%, whereas it augmented expression of the fast IIx and IIb MHCs in antigravity skeletal muscles. In fast muscles, SF caused subtle increases in the fast IIb MHC relative to the other adult MHCs. In contrast, TD prevented the normal expression of the fast MHC phenotype, particularly the IIb MHC, whereas TD maintained expression of the embryonic/neonatal MHC isoforms; this response occurred independently of gravity. Collectively, these results suggest that normal expression of the type I MHC gene requires signals associated with weight-bearing activity, whereas normal expression of the IIb MHC requires an intact thyroid state acting independently of the weight-bearing activities typically encountered during neonatal development of laboratory rodents. Finally, MHC expression in developing muscles is chiefly regulated by pretranslational processes based on the tight relationship between the MHC protein and mRNA data.

soleus; vastus intermedius; plantaris; tibialis anterior; gravity; mRNA; reverse transcriptase-polymerase chain reaction; myosin heavy chain

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IT IS WELL ESTABLISHED THAT the myosin heavy chain (MHC) molecule is the principal structural and regulatory protein that serves as the molecular motor to control the intrinsic contractile properties (force generation and shortening) of a muscle fiber (5, 31, 34). In adult rodents, at least four adult MHC genes have been identified in limb muscles, and these genes have been designated as the slow (type I), fast IIa, fast IIx, and fast IIb MHCs in increasing order of their putative ATPase activity and hence fiber-shortening properties (3, 5, 34). In addition, two developmental MHC isoforms have been identified in fetal/neonatal striated

muscles, and they have been designated as embryonic and neonatal MHCs (2, 11, 29, 30, 41).

In previous studies, we and others have shown that exposure to spaceflight or a microgravity environment of varying duration (days) induces muscle atrophy in both antigravity and locomotor muscles as well as a slow-to-fast contractile protein phenotype that is primarily manifested in the expression of hybrid fibers containing both slow and fast IIa and/or IIx MHC isoforms (3, 4, 8, 10, 22, 28, 35, 42, 46). Thus chronic weight-bearing activity appears to be essential in maintaining a bias to a slow contractile phenotype from both a quantitative and qualitative perspective in muscles heavily recruited to support normal weight-bearing movement activities.

In studies on developing rodent neonatal skeletal muscle, it is apparent that both antigravity (e.g., soleus) and locomotor (plantaris) muscles are in an undifferentiated state with regard to MHC gene expression following birth (2, 11, 30, 41); during the first 3–4 wk of development, these muscles undergo both rapid growth and a marked transformation in the expression of their respective adult MHC phenotypes (2). This involves a highly regulated process that appears to be closely coupled to the repression of neonatal and embryonic MHC isoforms that predominate MHC expression in the embryonic/neonatal stage (2, 11, 25). Thyroid hormone [3,5,3′-triiodothyronine (T_3)] is essential in this differentiation process in that it appears to play a critical role in the downregulation of the neonatal MHC isoform and in the concomitant de novo expression of the fast IIb MHC, which is the most predominant fast MHC isoform expressed in typical rodent fast-twitch muscles (3, 4, 10, 20, 22, 23). In the absence of T_3 availability, expression of the slow (type I) MHC gene is augmented in both antigravity and locomotor muscles of developing animals, while, at the same time, the embryonic and neonatal isoforms are retained, thereby maintaining the muscles used for locomotion in a partially undifferentiated state (2, 6, 17).

On the basis of the above observations on both adult and neonatal rodent skeletal muscle, we hypothesized the following concerning neonatal muscle development. *1*) Chronic weight-bearing activity, as occurs during the animal's normal developmental scheme, is essential for the normal expression of the slow myosin gene. *2*) Thyroid hormone is essential for the normal expression of the fast MHCs and in particular the fast IIb MHC gene; this requirement occurs independently of the

level of weight-bearing activity typically imposed on the muscle during neonatal development, i.e., animals not exposed to endurance training regiments (16). *3)* In the absence of both normal weight-bearing activity and an intact thyroid state, skeletal muscles remain in a partially undifferentiated state with regard to the adult pattern of MHC gene expression.

To test these hypotheses, we performed experiments as part of the National Aeronautics and Space Administration (NASA)/National Institutes of Health-sponsored Neurolab Mission in which neonatal rats of different age (i.e., 7 and 14 days of age at launch) and of different thyroid status [euthyroid and thyroid deficient (TD)] were exposed to the environment of spaceflight for 16 days aboard the space shuttle Columbia. This protocol enabled the neonates to encounter the space environment at critical time frames of non-weight-bearing activity during development when the skeletal muscle system normally is in a dynamic state of growth and differentiation. The findings reported herein are consistent with the above hypotheses and suggest that both chronic weight-bearing activity and an intact thyroid state are essential for the attainment of a normal adult MHC phenotype in slow-twitch antigravity and in fast-twitch locomotor skeletal muscles, respectively.

MATERIALS AND METHODS

Litter Formation and Experimental Design

As described in detail in the accompanying study (1), timed-pregnant female rats were obtained from Taconic Farms (Germantown, NY) and were housed initially in standard rodent cages in the vivarium at Kennedy Space Center in Florida. Shortly after birth, each litter was adjusted to an $n = 8$ pups and matched for gender distribution and designated as *1)* euthyroid vivarium control, *2)* euthyroid asynchronous ground control, *3)* TD vivarium control, *4)* TD asynchronous ground control, *5)* euthyroid flight-based, and *6)* TD flight based. The flight groups for this component of the project were launched into space at ~7 days of age. Additional information pertaining to the combining of experimental groups for data analysis and data presentation are covered in Table 1 of the accompanying study (1).

An additional three litters of animals from timed-pregnant rats were randomly assigned to experimental groups representing animals that were launched at ~14 days of age and were designated as *1)* euthyroid 14-day vivarium control, *2)* euthyroid 14-day asynchronous ground control, and *3)* euthyroid 14-day flight based. Because there were insufficient housing facilities for the older neonatal groups during spaceflight, ground-based and flight-based TD groups were not used in the experiments involving the older neonatal groups. In addition to the above experimental groups, three litters were also selected randomly, and these animals were killed on the day of launch (i.e., ~7 days of age) and used for baseline analyses. All experimental procedures were approved by both the NASA Institutional Animal Care and Use Committee (IACUC) and the University of California Irvine IACUC.

Details for inducing TD, cage configuration of the flight groups, and tissue processing protocols for this experiment have been described in detail in the accompanying study (1). In this study, we have focused on cardiac native isomyosin analyses as well as MHC protein and mRNA expression for the soleus, plantaris, vastus intermedius (VI), and tibialis anterior (TA) muscles.

Analytic Procedures For Cardiac Native Isomyosin Analysis

The ventricle samples were homogenized in 20 vol of a buffer containing 250 mM sucrose, 3 mM $MgCl_2$, 1 mM dithiothreitol, and 10 mM Tris·HCl, pH 7.4. The homogenate was diluted 10 times in a buffer consisting of 50% glycerol, 50 mM $Na_4P_2O_7$, 2.5 mM EGTA, and 0.5 mM β-mercaptoethanol (pH 8.8) and stored at $-20°C$. The separation of MHC isoforms was achieved by subjecting 10 µl of the stored dilute homogenate to nondenaturing PAGE for 20 h according to methods previously described in detail (21, 37) according to the procedures originally reported by Hoh et al. (24).

Analytic Procedures for Skeletal MHC Protein Analyses

The muscle sample was homogenized in 20 vol of a solution that contained 250 mM sucrose, 100 mM KCl, 5 mM EDTA, and 10 mM Tris·HCl, pH 7.0. The homogenate protein was diluted 10-fold with a storage buffer containing 50% glycerol, 50 mM $Na_4P_2O_7$, 2.5 mM EGTA, and 0.5 mM β-mercaptoethanol (pH 8.8) and stored at $-20°C$ until subsequent analysis for MHC protein determination.

Skeletal MHC protein isoforms were separated using an SDS-PAGE technique (39). This technique enabled us to separate both neonatal and embryonic MHCs in addition to the four adult MHCs typically expressed in rodent leg muscles as described in detail previously (see Ref. 2 and Fig. 1). The MHC protein isoform profile for each muscle was determined by laser scanning densitometry of the stained gel as described previously (2, 20, 23) using a Molecular Dynamics personal densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

MHC mRNA Analyses

The muscle sample was processed for total cellular RNA extraction by homogenization in the TriReagent (Molecular Research Center, Cincinnati, OH) according to the company's protocol. Total RNA was precipitated from the aqueous phase with isopropanol; after it was washed with ethanol, it was dried and suspended in a small volume of nuclease-free water. The RNA concentration was determined by optical density at 260 nm (using an equivalent to 40 µg/ml). The RNA samples were stored frozen at $-80°C$ until they were subsequently analyzed by RT-PCR technology (see below).

Analyses of MHC mRNA isoforms utilized a modification of an RT-PCR technique designed to quantitate relative amounts of MHC mRNAs representing the various MHC isoforms, as described in detail previously (43).

Reverse transcription. One microgram of total RNA was reverse transcribed for each muscle sample using the SuperScript II RT from GIBCO BRL and oligo(dT) primers according to the provided protocol. At the end of the RT reaction, the tubes were heated at 85°C for 5 min to stop the reaction and were stored frozen at $-80°C$ until used in the PCR reaction.

PCR primers and internal control fragment. The 5′ upstream primer was designed from a highly conserved region in all known rat MHC genes located at ~500–550 bp upstream from the stop codon. All seven MHC mRNA isoforms are identical in this region spanning ~35 nt. A 20-nt oligonucleotide was chosen of the following common sequence: 5′-AGAAGGAGCAGGACACCAGC-3′. The 3′ MHC isoform-specific oligonucleotides used in the PCR reaction were basically the same as those used previously (43) except the embryonic oligo sequence was as follows: CCCTCAC

CAGGAGGACATGC. A correction in the sequence (underlined) was included based on the mRNA sequence published in GenBank (accession X04267). The internal fragment also was modified to be used as a competitor for all known MHC isoforms. With the use of the sequence information, the internal fragment was made shorter and the new common primer sequence was ligated at the 5′ end, whereas the neonatal- and embryonic-specific antisense primers were linked in tandem to its 3′ end already containing types I, IIa, IIx, and IIb (43).

PCR. Each RT reaction was diluted 20-fold with nuclease-free water and mixed with an equal volume of control fragment at the appropriate dilution (~1 amol/µl). Two microliters of this mixture were used for the 25-µl PCR reactions. The PCR reaction was carried out in the presence of 2 mM $MgCl_2$ using standard PCR buffer (GIBCO), 0.2 mM dNTP, 1 µM primers, and 0.75 unit of DNA *Taq* polymerase (GIBCO). Amplifications were carried out in a Stratagene Robocycler with an initial denaturing step of 3 min at 95°C, followed by 25 cycles of 45 s at 95°C, 50 s at 52°C, 50 s at 72°C, and a final step of 3 min at 72°C. The number of cycles was optimized so that the amplified signal was still in the linear range of the semilog plot of the yield expressed as a function of the number of cycles. PCR products were separated on a 1.5% agarose gel by electrophoresis (see Fig. 1), and signal quantification was done as reported previously (43). A correction factor was calculated for each band based on its size to normalize the intensity of the ethidium bromide staining to account for the different sizes of the control synthetic fragment. With the use of this method, each specific MHC mRNA isoform expression is calculated as relative to the total MHC mRNA pool.

Statistical Analyses

All data are reported as means ± SE. Statistical differences between the groups were tested using a one-way ANOVA procedure; when differences were detected for a given variable, a Newman-Keuls post hoc test was used. All statistical analyses were performed using a computer software package (Prism, GraphPad Software). Linear regression analyses between MHC mRNA and protein values and correlations between neonatal/embryonic vs. adult MHC expression were performed using a Prism-GraphPad statistical analyses software package. Statistical significance was set at $P < 0.05$.

RESULTS

General Observations

In this 16-day flight experiment, for reasons not fully understood at the present time, ~40 of the 80 younger neonates that were flown in the research animal holding facility cages did not survive the 16-day mission, with many of them expiring after ~7–8 days in space. As shown in Table 2 of the accompanying study (1), two euthyroid flight neonates in the litter assigned to this experiment were lost. In contrast, two TD flight rats were lost during spaceflight and two additional TD flight rats were deemed by the NASA veterinarian to be in poor health and thus were excluded from our analyses. The younger animals that survived the effects of spaceflight appeared to be in good health and were nurtured by their dams, based on observations by the attending flight veterinarian. None of the older (*day 14*)

neonatal rats expired during spaceflight, and none of the animals comprising the ground-based control groups was observed to be in ill health. Despite these reductions in animal number assigned to this project, we were able to obtain sufficient data to clearly differentiate responses in the variables investigated due to both spaceflight and altered thyroid state. Also, as discussed further herein, as well as in the accompanying study (1), we are confident that the euthyroid and TD flight animals were not nutritionally compromised to an appreciable extent so as to invalidate the findings of this study. This is based on both direct and indirect evidence (see below), even though no direct data were obtained on the caloric balance of the flight- vs. ground-based neonatal groups. Furthermore, results obtained on the two ground-based groups used in the study for both euthyroid and TD animals were nearly identical for the variables investigated so that these groups were combined to simplify data presentation.

Cardiac Isomyosin Patterns

At the time of launch (e.g., 7 days of age), the α-MHC isoform comprised 69% of the cardiac MHC pool (Table 1); by 23–30 days of age, the relative content of the α-MHC increased to 95–98% of the MHC pool in both the flight- and ground-based euthyroid groups. These values are typical for hearts of euthyroid, nutritionally fortified rodents in this age group (2, 14). In contrast, in both the flight- and ground-based TD groups, the pattern was essentially reversed, as the α-MHC contributed only ~5% of the total MHC pool. These results, in combination with the findings on body and muscle weights in the accompanying paper (1), as well as the close approximation of the body weight and cardiac and skeletal isomyosin (including circulating T_3 data) reported in a previous ground-based study (2) relative to the data reported in the combined papers herein, collectively suggest that the thyroid state of the flight- and ground-based TD groups was significantly compromised to a similar degree. Also, because the TD flight group received the antithyroid drug via the mother's milk, it would appear that these neonates were not nutritionally deprived. Previously, we and others have reported that cardiac MHC profiles are a sensitive marker of both the thyroid status (2) and nutritional

Table 1. *Heart MHC protein isoform expression as determined by native gel electrophoresis*

	n	α-MHC, %	β-MHC, %
Basal	8	69 ± 1†	31 ± 1†
NC7 ground	16	97 ± 0.5	3 ± 0.5
NC7 flight	6	98 ± 1	2 ± 1
TD7 ground	16	7 ± 1.6†	93 ± 1.6†
TD7 flight	4	6 ± 0.2†	94 ± 0.2†
NC14 ground	12	95 ± 0.6	5 ± 0.6
NC14 flight	6	96 ± 1	4 ± 1

Values are means ± SE; n = no. of rats. MHC, myosin heavy chain; NC7 ground, euthyroid vivarium control (VC) + euthyroid asynchronous ground control (AGC); TD7 ground, thyroid-deficient VC + thyroid-deficient AGC; NC14 ground, euthyroid 14-day VC + euthyroid 14-day AGC (the older neonates). † $P < 0.05$ vs. NC7 ground.

Table 2. *Soleus MHC protein and mRNA isoform expression*

	n	Embryonic	Neonatal	Type I	Type IIa	Type IIx	Type IIb
Basal	8						
Protein		27.3 ± 1.4†	25 ± 9†	45.2 ± 8†	1.1 ± 0.2†	ND	1.5 ± 0.5
RNA		37.8 ± 1.8†	26 ± 2.5†	28.2 ± 2†	7.3 ± 1.5†	ND	0.3 ± 0.1
NC7 ground	16						
Protein		ND	ND	78.8 ± 1	20.5 ± 0.8	0.7 ± 0.3	ND
RNA		17.4 ± 1	0.5 ± 0.2	49.1 ± 2	27.8 ± 2	4.5 ± 1.4	0.7 ± 0.2
NC7 flight	6						
Protein		ND	3.6 ± 0.7	35.5 ± 4*	41.8 ± 5*	16.4 ± 2*	2.7 ± 1*
RNA		6.2 ± 0.8*	2.5 ± 0.6	25.3 ± 2*	26.2 ± 2	36.2 ± 3*	3.8 ± 1.2*
TD7 ground	16						
Protein		9.6 ± 0.4†	6.2 ± 0.6†	83.8 ± 1	0.8 ± 0.1†	ND	ND
RNA		15.6 ± 1.3	8.8 ± 1.7†	74.2 ± 1.7†	0.2 ± 0.1†	ND	0.9 ± 0.4
TD7 flight	4						
Protein		8.4 ± 1.2	14.5 ± 1.8*†	75.8 ± 3	0.9 ± 0.3†	ND	0.5 ± 0.5
RNA		22.5 ± 2.5*	36.5 ± 5*†	38.5 ± 6*†	1.3 ± 0.6†	0.5 ± 0.2	0.8 ± 0.3
NC14 ground	12						
Protein		ND	ND	78.8 ± 1	20.0 ± 1	0.7 ± 0.2	1.1 ± 1.1
RNA		14.3 ± 1.3	0.4 ± 0.2	55.1 ± 3†	23.8 ± 1	4.7 ± 1.2	1.4 ± 0.6
NC14 flight	6						
Protein		ND	ND	25.1 ± 2*	31.8 ± 1*†	40.5 ± 2*†	2.6 ± 0.8†
RNA		4.3 ± 0.8*	0.5 ± 0.2	19.2 ± 1*†	22.8 ± 2	49.7 ± 2*†	3.3 ± 1.1

Values are means ± SE; n = no. of rats. MHC data are expressed as percentage of total MHC pool. ND, not detected. * $P < 0.05$, ground vs. flight; † $P < 0.05$ vs. NC7 ground.

(energy consumption) status (13, 14, 21, 37) of neonatal, young, and adult rodents. That is, both TD and caloric deprivation markedly shift the cardiac MHC profile from one of marked α-MHC predominance (>85%) to a pattern in which, in the case of TD, the β-MHC is almost exclusively expressed (25), whereas, under caloric deprivation, the β-MHC can comprise as much as 50–60% of the total cardiac MHC pool (21, 37). Clearly, this sensitive marker of both the thyroid state and nutritional status strongly indicates that the findings reported herein on the euthyroid flight animals relative to their ground control counterparts were unlikely impacted to any appreciable extent by either the nutritional and/or thyroid status of these particular experimental groups (see DISCUSSION for additional comments).

Skeletal Isomyosin Profiles

Baseline values. Baseline analyses on 7-day-old neonates were performed on each of the muscles selected for study (see Tables 2–5). MHC profiles for both the soleus and VI muscles clearly show that they are in an undifferentiated state relative to their respective adult patterns, since ~40–50% of the MHC pool was composed of embryonic/neonatal isoforms (Tables 2 and 3), and, in the case of the soleus muscle, the type I MHC isoform was <60% of the values typically seen in the

Table 3. *Vastus intermedius MHC protein and mRNA isoform expression*

	n	Embryonic	Neonatal	Type I	Type IIa	Type IIx	Type IIb
Basal	8						
Protein		26.0 ± 1†	17.0 ± 3†	51.1 ± 3†	2.6 ± 0.2†	ND†	3.3 ± 0.4
RNA		32.4 ± 2†	32.6 ± 2†	23.4 ± 2†	8.6 ± 1.4†	1.8 ± 0.5†	1.3 ± 0.2†
NC7 ground	16						
Protein		ND	0.6 ± 0.2	40.6 ± 2	39.2 ± 1	13.1 ± 2	6.5 ± 1.0
RNA		12.7 ± 2	0.4 ± 0.1	35.8 ± 2	28.4 ± 1	16.8 ± 2	5.9 ± 1
NC7 flight	6						
Protein		ND	2.3 ± 0.2	45.7 ± 2	28.0 ± 2*	15.0 ± 1	9.1 ± 1
RNA		3.5 ± 0.4*	0.7 ± 0.2	27.8 ± 1	15.7 ± 1*	30.3 ± 2*	22.3 ± 2*
TD7 ground	16						
Protein		12.6 ± 1†	7.6 ± 0.7†	74.7 ± 2†	1.6 ± 0.4†	0.4 ± 0.2†	3.1 ± 0.3
RNA		22.9 ± 2†	15.1 ± 2†	52.5 ± 3†	6 ± 1.2†	1.8 ± 0.3†	1.8 ± 0.1†
TD7 flight	4						
Protein		6.2 ± 0.6*†	10.9 ± 1†	75.4 ± 1†	2.3 ± 0.3†	ND†	5.2 ± 0.3
RNA		26.5 ± 1†	23.3 ± 0.6*†	39.8 ± 3*	6 ± 1.5†	2.5 ± 0.3†	2.3 ± 0.3
NC14 ground	12						
Protein		ND	ND	46.3 ± 3	36.0 ± 1	11.5 ± 2	6.2 ± 1.4
RNA		11.5 ± 1	0.25 ± 0.1	38.9 ± 1	29.3 ± 1	15.6 ± 2	4.3 ± 0.8
NC14 flight	6						
Protein		ND	ND	34.4 ± 2*	20.0 ± 1*†	33.5 ± 2*†	12.0 ± 1*†
RNA		4.3 ± 0.5*†	0.67 ± 0.2	25.8 ± 2*†	18.3 ± 1*†	35.7 ± 1*†	15.2 ± 3*†

Values are means ± SE; n = no. of rats. MHC data are expressed as percentage of total MHC pool. * $P < 0.05$, ground vs. flight; † $P < 0.05$ vs. NC7 ground.

1 and 2). This was offset by significantly greater than normal expression of the fast type IIa and IIx MHC isoforms, as well as a slight augmentation of trace levels of the type IIb MHC. This repression in type I MHC isoform expression at the protein level was also observed at the mRNA level (Table 2 and Fig. 2). In contrast, although the developmental process to increase fast type IIa expression in the normal ground control animals appeared to be associated with augmentation of pretranslational events (mRNA), the further increase in the relative expression of this isoform in response to spaceflight appeared to be under the greater control of translational events (Table 2 and Fig. 2). The mechanism for this unique pattern of type IIa MHC protein regulation in response to spaceflight is unclear at the present time. Also, the marked increase in the nearly de novo expression of the IIx MHC in the soleus muscle of the flight groups was clearly associated with pretranslational events based on the marked increase in the type IIx mRNA levels in the flight groups relative to ground control values (Table 2 and Fig. 2). An interesting feature of the mRNA data was the retention of embryonic expression at the mRNA level in the 7-day-old normal control (NC7) and 14-day-old normal control (NC14) euthyroid ground groups, although both our gel system and a more sensitive Western blot technique using embryonic/neonatal MHC antibodies (2) could not detect its expression at the protein level (Table 2). Interestingly, we have recently observed that embryonic mRNA is expressed in soleus muscles of much older adult rats, although no embryonic MHC protein is expressed (12). The reason for this uncoupling of expression of embryonic MHC mRNA vs. protein is unknown at the present time.

In contrast to the soleus, a slightly different pattern of response to spaceflight occurred for the VI muscle involving the older neonatal group in that both the slow type I and fast type IIa MHC were repressed, whereas that of the IIx and IIb MHCs were significantly increased relative to the ground control group (Table 3).
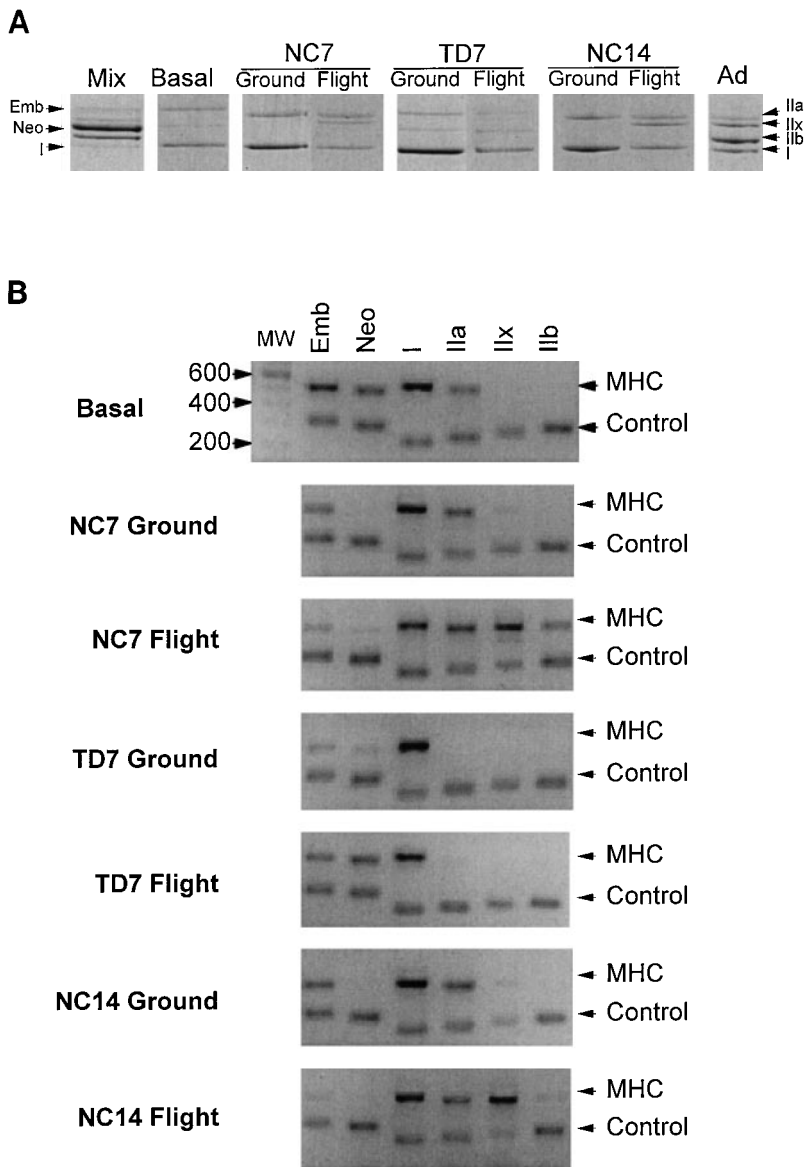

Fig. 1. *A*: Coomassie blue-stained myosin heavy chain (MHC) protein isoforms of representative soleus muscles of the different groups as separated by SDS-PAGE. Although these individual samples were run on separate gels, each gel contained its own markers for all 7 individual MHC isoforms for correct band identification. Mix, MHC isoforms in protein extract from a mix of adult and neonatal skeletal muscles, which contained all 7 MHC isoforms. Ad, MHC isoforms in protein extract from adult skeletal muscles, which contained all 4 adult MHC isoforms. Emb, embryonic; neo, neonatal MHC isoform. *B*: soleus total RNA was subjected to RT-PCR, and reaction products were separated by electrophoresis on 1.5% agarose gel and stained with ethidium bromide. Shown are representatives of the different groups. In each PCR reaction, 2 products are coamplified: *1*) MHC cDNA from the total RNA sample subjected to RT, ranging from 516 to 545 bp in size, and *2*) control, which is the externally added synthetic fragment and which is flanked by identical primer sequences as those used for the different MHC amplifications (see Ref. 39 for details). Control fragment amplification product size ranges from 224 (I) to 324 bp (emb) with 20-bp increments in the following order: I, IIa, IIx, IIb, neo, and emb. MW, DNA molecular weight marker (100-bp ladder). NC7, 7-day-old normal control (euthyroid); TD7, 7-day-old thyroid deficient (hypothyroid); NC14, 14-day-old normal control.
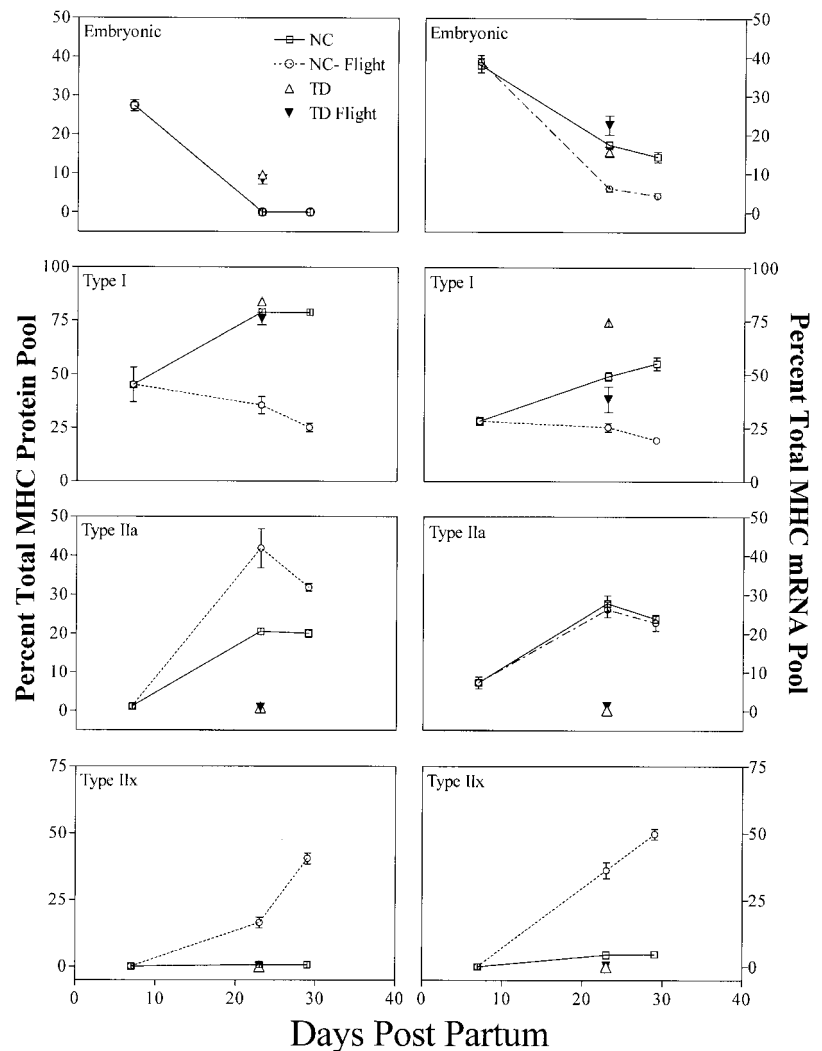
Fig. 2. Temporal changes in relative MHC protein and mRNA expression of selected isoforms in the soleus muscle. Effects of TD and 16 days of spaceflight are shown.

However, in the VI muscle of the younger neonatal animals, there were little differences in MHC isoform expression in flight vs. ground controls (Table 3). This unexpected observation is difficult to explain but might be due to a surprising lower level of expression of type I MHC protein in the ground control group relative to that seen in the 7-day-old basal group (Table 3). That is, it seems reasonable to assume that the relative level of type I MHC expressed in the VI at 23 days of age should approximate that seen in the basal state, i.e., at 7 days of age (Table 3). Clearly, more research is needed to unravel this apparent discrepancy. However, both the developmental patterns and the spaceflight-induced transformations in MHC expression in the VI muscle appeared to be largely regulated by a coupled pretranslational/translational process, based on the agreement in the mRNA and protein patterns of response across the experimental groups (Table 3).

Fast-twitch muscles. By 23–30 days of age, the adult fast MHC profile was attained, since the neonatal/embryonic isoforms were not detected at the protein level and the fast MHCs (IIa, IIx, and IIb) reached peak levels of expression in the ground control animals (Tables 4 and 5).

In the plantaris muscle, a fast-twitch synergist to the soleus muscle, exposure to spaceflight modulated the MHC developmental profile by *1*) potentiating expression of the fast IIb isoform and *2*) slightly decreasing the relative content of the IIx, IIa, and slow (type I) MHCs (chiefly in the younger neonatal flight group; Table 4 and Fig. 3). These responses at the protein level were essentially mirrored by the mRNA profiles.

In the TA, which is a non-weight-bearing ankle flexor used in locomotion, spaceflight exerted a subtle impact, chiefly in the younger neonatal flight group, by creating a greater bias to the fast type IIx and IIa MHCs (e.g., decreasing relative IIb content) compared with the ground control group (Table 5). No differences were noted between flight vs. ground controls for the older neonatal groups, and, as noted for the plantaris, mRNA responses were in general agreement with those obtained at the protein level.

Effects of Hypothyroidism and Interactions with Spaceflight

In general, TD slowed and/or prevented the transition to the adult MHC phenotype typically seen in both
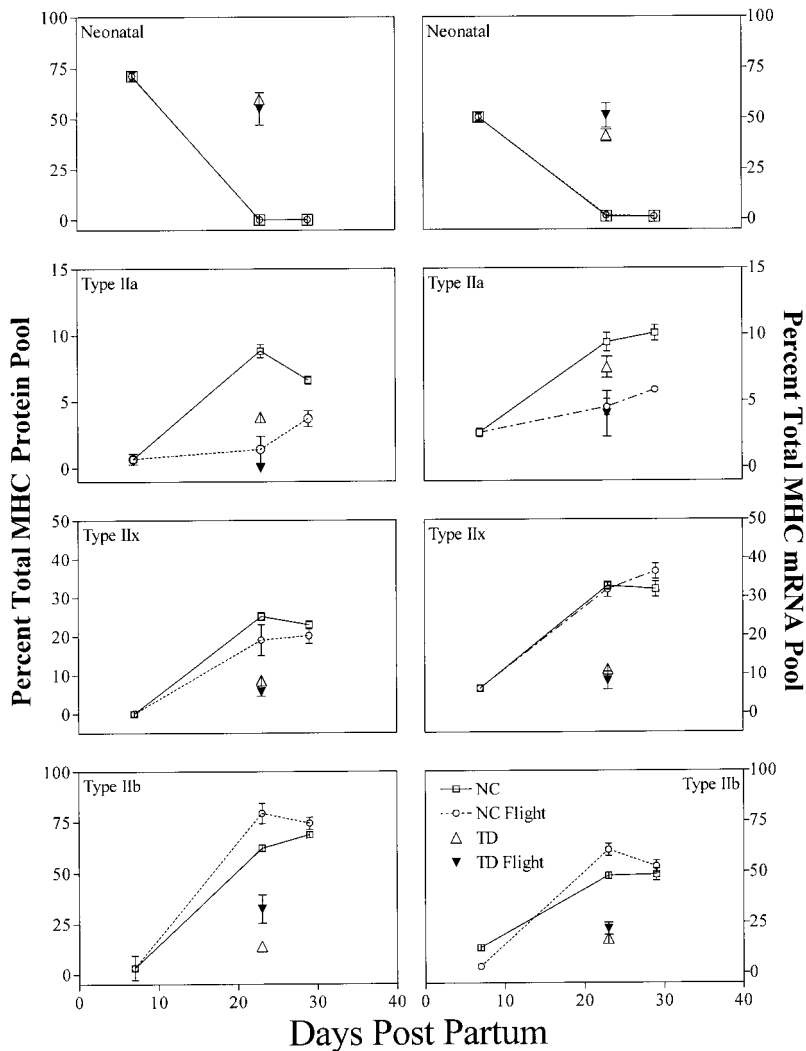
Fig. 3. Temporal changes in relative MHC protein and mRNA expression of selected isoforms in the plantaris muscle. Effects of TD and 16 days of spaceflight are shown.

the slow-twitch and fast-twitch muscles of euthyroid rats (Tables 2–5). This TD effect occurred irrespective of loading state, by maintaining significant levels of expression (protein and mRNA) of the embryonic and neonatal MHC isoforms across the spectrum of muscles examined (Tables 2–5).

Furthermore, in the slow-twitch muscle types (soleus and VI), TD effectively blunted the spaceflight-induced repression of type I MHC gene expression such that the type I MHC values of the TD flight group in the soleus were in line with those seen for both the younger and older euthyroid ground-based controls (Table 2 and Fig. 2). In the VI, there was an equivalent augmentation in type I MHC expression in both the TD flight- and ground-based groups compared with the euthyroid ground-based controls (Table 3 and Fig. 2). These responses on slow-twitch muscle were influenced by pretranslational processes based on the mRNA profiles (Tables 2 and 3). Thus TD exerted a powerful influence on MHC expression in the slow-twitch muscle types by *1*) uniquely augmenting slow MHC gene expression, *2*) blunting the normal repression of neonatal/embryonic gene expression, *3*) inhibiting the flight-associated increase in type IIx MHC expression, and *4*) inhibiting

both the developmental- and flight-induced increases in type IIa MHC expression (Table 2 and Fig. 2).

In both the plantaris and TA muscles, TD, in addition to maintaining the expression of the neonatal/embryonic MHC isoforms, primarily exerted its impact on the transition to the adult phenotype by significantly inhibiting what appears to be a neonatal-type IIb MHC transition (see below) in MHC phenotype that is likewise regulated, in part, by pretranslational processes (Tables 4 and 5). Moreover, it appears that, in terms of the interaction of TD and the environment of spaceflight on type IIb MHC expression in developing muscle, TD appears to be only partially effective in inhibiting the expression of IIb MHC in the flight group vs. that seen for the ground-based group (Tables 4 and 5 and Fig. 3).

Correlations Impacting MHC Expression Across Experimental Groups

We have noted that there appeared to be relatively good agreement between the mRNA and protein expression patterns of MHC isoforms as examined in the different groups. To test this relationship further, we

performed regression analysis of the mRNA vs. the protein values for a given adult MHC across all the muscles and experimental groups examined in this study. As presented in Fig. 4, we found very high correlation between the two variables, with r values ranging from 0.79 to 0.93, which were highly statistically significant. These results reflecting a tight relationship strongly indicate that the changes in MHC protein expression depend significantly on the coexisting mRNA levels. Thus these findings provide convincing information that the regulation of the adult MHC genes is strongly influenced by pretranslational events. Also, the database generated herein allowed us to further examine the stoichiometry in neonatal/embryonic-to-adult MHC transformations across the various muscles. As shown in Fig. 5, we observed a very high inverse correlation ($r = -0.96$) between the downregulation of the neonatal MHC and the upregulation of the type IIb MHC across the TA and plantaris muscle pools, i.e., those fast-twitch muscles that initially express a high relative content of the neonatal isoform (Tables 3 and 4). Furthermore, it would appear that such a coupling between neonatal and IIb MHC transitions can only apply to fast-twitch muscles, since the soleus, which expresses some neonatal MHC in the early stages of development, does not normally express measurable amounts of the type IIb MHC in the adult/differentiated state. On the other hand, because the embryonic MHC was equally well expressed early on across all the muscles (see basal values in Tables 2–5), it appeared that the repression of this isoform was not linked to any specific adult MHC isoform in the hindlimb musculature. Consistent with this notion, we observed a reasonably good negative correlation ($r = -0.63$; $P < 0.001$) when the embryonic MHC relative expression was correlated to the combined expression of the type I, IIa, and IIx isoforms in fast-twitch muscle types (TA and plantaris, Fig. 5). The correlation was even stronger when data from the soleus and VI were analyzed (Fig. 5). These relationships suggest that a large pool of the fibers comprising the hindlimb musculature, which initially express the embryonic isoform, is not necessarily committed to a single isoform in particular (as appears to be the case for the neonatal isoform in fast-twitch muscles). Rather, these fibers appear to give rise to a variety of MHC-expressing fiber types, depending on the environmental conditions imposed and the type of muscle involved. This notion is further illustrated by the fact that, in the normal soleus, there was a good stoichiometry between the downregulation of embryonic MHC and the upregulation of type I MHC (Table 2). In the VI, the stoichiometry favored a coupling between embryonic and the type IIa MHC (Table 3), whereas, in the plantaris muscle, there appeared to be a coupling process likely involving the type IIx MHC (Table 4).

DISCUSSION

The environment of spaceflight (often referred to as microgravity) imposes a unique chronic condition on the contractile apparatus of skeletal muscle in that relatively little force is generated when the muscle is activated to create movement. In studies performed largely on adult rodents, spaceflight of varying duration (6–14 days) induces both a general atrophy response across the musculature of the lower limbs (1, 4, 8, 10, 22, 28, 35, 46) and a transformation in contractile

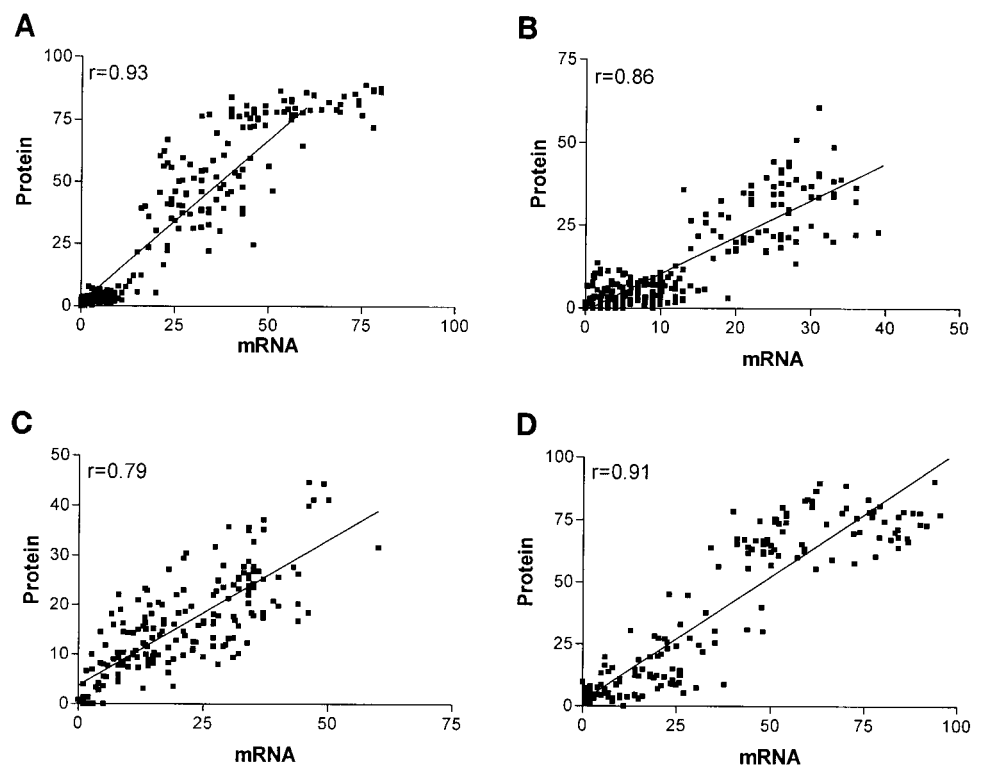Fig. 4. MHC isoform protein-mRNA relationships using data from all the muscle groups [soleus, vastus intermedius (VI), plantaris, tibialis anterior (TA)], including all the experimental groups. A: type I MHC. B: type IIa MHC. C: type IIx MHC. D: type IIb MHC. Lines were generated by linear regression analysis (Prism GraphPad). The r value is the correlation coefficient between the 2 variables. $P < 0.05$ for all 4 isoforms.
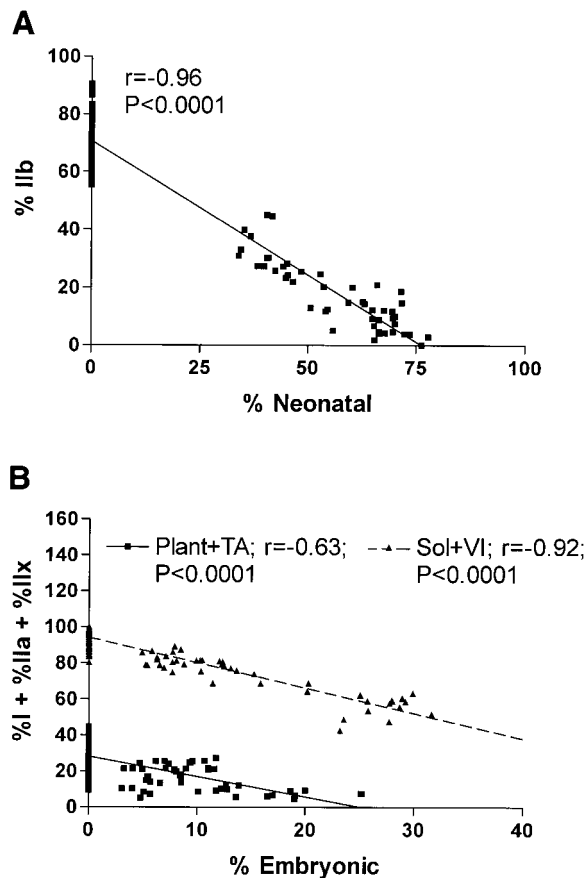
Fig. 5. Inverse relationships between developmental (neonatal and embryonic) and adult (I, IIa, IIx, and IIb) MHC protein isoforms in developing rat skeletal muscles. *A*: data from plantaris (plant) and TA muscles. *B*: regression lines were distinguished between fast-twitch (plantaris and TA data) and slow-twitch [soleus (sol) and VI data] skeletal muscles. All correlations were statistically significant.

protein phenotype whereby a large percentage of the fibers (which originally expressed almost exclusively slow type I MHC) is transformed to coexpress both fast and slow MHCs (8, 10, 22). These transformations can be closely mimicked by the ground-based model of hindlimb suspension in which nearly identical changes to those summarized above for spaceflight also occur during the same time frame (3, 4, 7, 9, 13, 18, 22, 23). In the context of the latter observations involving the hindlimb suspension model, additional studies have shown that *1*) changes in type I and IIb MHC phenotype are strongly regulated by transcriptional/pretranslational processes (18, 26, 36) and *2*) the thyroid state (e.g., hypothyroidism) can dominate that of unloading in controlling the bias of MHC phenotype when the two interventions are imposed simultaneously on an adult rodent (13).

The experiments reported herein provided a rare opportunity to study MHC gene expression, in the context of both spaceflight and thyroid state, from a different perspective in which the target muscles were manipulated by these imposed variables at a time when the limb muscles were still phenotypically in an undifferentiated state (Tables 2–5). The findings concerning MHC gene expression and muscle mass [as reported in

the accompanying study (1)] obtained on those neonatal groups that were exposed to spaceflight at critical periods during development are consistent with the above-mentioned findings reported on adult rats except that the observations on the former appear to be quantitatively more dramatic, given the critical stage of muscle development at which the manipulations were imposed.

For example, the effects of spaceflight on MHC gene expression were chiefly manifested as a marked repression of the slow, type I MHC gene relative to the ground control response, and this occurred for both the "younger (NC7)" and "older (NC14)" neonatal groups (Tables 2–5 and Figs. 2–4). Furthermore, this process was seen for the most part across all muscles that express this gene (with the impact being greater in so-called antigravity muscles, i.e., soleus and VI), and the response appears to be strongly regulated by pretranslational events (Tables 2–5 and Figs. 2–4). In our view, as a result of exposure to spaceflight, both the soleus and VI muscles cannot be considered as typical slow-twitch muscles in the euthyroid flight neonatal animals, since the slow MHC accounts for only 20–40% of the MHC pool in these prototype muscles. Furthermore, in a typical fast-twitch muscle normally used in ground support/locomotion (e.g., plantaris), the adaptation is more subtle and is manifest chiefly by a further bias to expression of the fastest MHCs (i.e., IIb MHC). This latter observation was most pronounced in the younger neonatal flight group. Together, these results suggest that, in the absence of normal weight-bearing and locomotor activity under the loading conditions routinely imposed by gravity, the contractile phenotype of the musculature becomes clearly biased to a fast(er) MHC profile. The exception to this generality is seen, however, in non-weight-bearing muscles such as the TA in which both muscle mass and contractile protein phenotype were not compromised to the same degree as in those muscles routinely used in weight-bearing/locomotor activities during development in the spaceflight environment (Table 5).

Although one might argue that the effect of spaceflight on MHC isomyosin gene expression in neonatal rats is compromised by the fact that a significant number of animals were lost relatively early on in the mission, due possibly to the inability of the dams to nurture their litters as described in the accompanying study (1), we feel that it is unlikely that nutrition per se is responsible for the contrasting responses reported herein between the flight vs. control young animals for the following reasons. First, Dowell and Martin (14) have reported that, when neonatal rats are nutritionally deprived via the model of markedly increasing the size of the litters ($n = 16$ vs. $n = 8$ for controls), the rate of body and muscle growth is correspondingly retarded by ~30% at the time of death (e.g., 21 days of age, which is similar to the time frame used in the present study). In these slower-growing neonates, the authors reported a significant reduction in the relative content of the α-MHC isoform in the heart relative to the normal-growing control neonates; the authors further showed

that the perinatal nutritional reduction had an effect on reducing α-MHC expression that was independent of neonatal thyroid status (14). In the present study, although body growth of the younger euthyroid flight groups was $\sim$50% that of their euthyroid ground controls (1), there was no evidence that α-MHC expression was compromised (Table 1). Second, the differences in the cardiac and skeletal isomyosin data of the flight vs. ground controls of the older neonatal experimental groups were quantitatively and qualitatively quite similar to the differences seen in the younger flight vs. ground control groups (Tables 1–5). Given the fact that the older neonatal flight animals showed little or no evidence of nutritional imbalance, as seen in their body and muscle weight profiles relative to ground controls (1), it would appear that the younger euthyroid neonatal flight animals were not appreciably compromised nutritionally to impact the MHC gene expression beyond that seen as a result of the separate and combined interventions of spaceflight and TD. Finally, because both the ground control and TD flight neonatal groups were made hypothyroid by the vehicle of the mothers milk and given the close agreement of these two groups across the data spectrum examined, it is also likely that the TD flight animals were not appreciably energy compromised as well. Otherwise, we would have anticipated lesser effects due to TD on MHC gene expression in the TD flight group relative to the TD ground controls.

Despite these adaptations in response to the spaceflight environment, an intact thyroid state appears to be a key regulator of MHC phenotype during normal muscle development irrespective of gravity. This is seen by the dominating influence that TD had concerning 1) the enhancement of type I MHC protein expression in the soleus and VI muscles of both the ground-based and flight-based groups whose profiles were similar in the hypothyroid state (Tables 2 and 3) and 2) repression of the "developmental switch," whereby neonatal and embryonic MHC expression is repressed and rapidly replaced by adult MHCs (Tables 2–5 and Figs. 2 and 3). Thus the effects of TD are quite global and can dominate the control of MHC expression in the developmental cascade. With the exception of enhancing regulation of the type I gene in the neonatal state, its effect on neonatal muscle is to maintain the muscle largely in an undifferentiated state, i.e., biased to a neonatal/embryonic MHC phenotype.

What factors account for the divergent effects of muscle unloading and thyroid hormone on the regulation of type I MHC gene expression in developing skeletal muscle? Because the findings reported herein and elsewhere (10, 18, 22, 23, 25, 32, 40, 44) clearly suggest that the regulation of this gene in striated muscle (heart and skeletal) involves processes manifested at the transcriptional/pretranslational level, we have initiated studies to examine its regulation in vivo using direct transfection of DNA promoter-reporter constructs into target muscles (18, 44). In the context of these experiments, a putative thyroid response element (TRE) is thought to be located in the basal promoter of the type I MHC gene (15). Mutating this element in both full-length and shorter (e.g., -408) promoter fragments essentially annihilates activity of the promoter when expressed in both cardiac and soleus skeletal muscle (Ref. 44 and unpublished observations). We interpret this finding to suggest that this TRE (possibly in combination with other half-site TREs) is essential for normal type I MHC transcriptional activity and that, when the TRE is bound by thyroid receptors and thyroid receptor auxiliary proteins in the absence of T_3, transcriptional activity is enhanced (19, 32, 44, 45). When T_3 is present in sufficient abundance, it is thought to cause a change in configuration of the basal transcription machinery, thereby inhibiting type I promoter activity, as seen during hyperthyroidism (19, 45). Also, we have shown that the type I MHC promoter becomes inhibited when transfected into the soleus muscle in response to hindlimb unloading (18). This inhibition is postulated to be under the control of a negative element, designated as βe1 (40), which is postulated to become activated in the unloaded state (18). Furthermore, mutation of this element in a short -408 fragment renders it unresponsive to unloading compared with the wild-type fragment (unpublished observations). Together, these findings suggest that there are key *cis* regulatory elements and corresponding *trans*-acting factors that provide sensitive control of the type I promoter in response to a thyroid state as well as mechanical unloading, consistent with the findings reported herein. The fact that TD may dominate that of unloading in the control of type I MHC gene expression may be because one of the TREs may be located in a more strategic location in the promoter to thus dominate the basal transcriptional activity of the gene under different physiological states, e.g., TD vs. spaceflight.

Information concerning regulation of the IIb MHC gene is much less understood in response to altered thyroid state and mechanical activity. Recently, Swoap (36), using deletion analysis technology, reported that only 295 bp in the proximal IIb promoter region upstream of the initiation start site is required to induce responsiveness of the IIb MHC gene in normal fast-twitch muscle and in soleus muscle in response to hindlimb unloading. Interestingly, Sachs et al. (33) demonstrated that a -1433-bp IIb MHC promoter activity was upregulated in *Xenopus* tadpole dorsal muscle 49 h after treatment with T_3, a response that mimicked the endogenous IIb MHC gene response. This response has been attributed to potential TRE half sites located on the IIb promoter (33). Sequence analyses of the mouse IIb MHC promoter (38) have revealed several half-site TREs in the proximal region of the IIb MHC promoter, which may play a pivotal role concerning the endogenous IIb MHC gene regulation. Thus, during neonatal development, T_3, by both 1) its direct effect on TREs as mentioned above and 2) an indirect influence, mediated by transcription factors such as MyoD, which has TREs in its promoter (27), could exert a powerful effect on transcriptional events involving IIb MHC gene expression in skeletal muscle, as the find-

ings reported herein and elsewhere strongly suggest (18, 36).

In summary, the environment of spaceflight provided a unique opportunity to interact the separate and combined effects of limb unloading and TD on muscle growth and the development of MHC gene expression in key limb muscles of neonatal rodents. The results reported herein and in the accompanying study (1) clearly suggest that chronic weight-bearing activity is essential for *1*) normal muscle growth as likely mediated by systemic and local mechanisms involving the T_3-growth hormone-insulin-like growth factor I axis and *2*) the establishment of slow motor genes in muscles that are essential for antigravity function and locomotion. These adaptations to weight-bearing stimuli enable the slower twitch muscles to effectively oppose gravity despite a dominant effect of T_3, which is necessary for transforming the fast skeletal musculature to attain an adult fast MHC phenotype. This latter transformation process appears to occur independently of the weight-bearing activity that the cage-confined neonatal rat typically encounters during development. However, the regulatory mechanisms controlling MHC gene expression during development and in response to loading state remain to be elucidated.

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