Microgravity associated with spaceflight induces numerous physiological changes in skeletal muscle. These include fiber type conversion which has a profound impact on skeletal muscle function. The fast isoforms of contractile proteins, such as myosin heavy chain (MHC) IIb, are upregulated during muscle unloading. This process results in a diminished capacity for low-intensity endurance work and could ultimately result in an increased rate of fatigue, as well as lowering the overall productivity and comfort of astronauts during prolonged missions. The precise signals which direct muscle protein isoform switching during unloading need to be defined in order to develop successful countermeasures to spaceflight-induced skeletal muscle dysfunction.

Our stated specific aims for this project were: 1) Identify the region(s) of the mouse MHC IIb promoter necessary for in vivo expression in mouse fast-twitch muscle, and 2) Identify the region(s) of the mouse MHC IIb promoter responsive to immobilization in mouse slow-twitch muscle in vivo. We sought to address these specific aims by introducing various MHC IIb reporter gene constructs directly into the tibialis anterior and gastrocnemius muscles of living mice. Although the method of somatic gene transfer into skeletal muscle by direct injection has been successfully used in our laboratory to study the regulation of the skeletal alpha actin gene in chicken skeletal muscle, we had many difficulties utilizing this procedure in the mouse. Because of the small size of the mouse soleus and the difficulty in obtaining consistent results, we elected not to study this muscle as first proposed. Rather, our MHC IIb promoter deletion experiments were performed in the gastrocnemius. Further, we decided to use hindlimb unloading via tail suspension to induce an upregulation of the MHC IIb gene, rather than immobilization of the hindlimbs via plaster casts. This change was made because tail suspension more closely mimics spaceflight, and this procedure in our lab results in a smaller loss of overall body mass than the mouse hindlimb immobilization procedure. This suggests that the stress level during tail suspension is less than during immobilization.

Unfortunately, our results were unanimously negative. In other words, we were unable to demonstrate that the activity of any of the promoter lengths studied (-1340, -675, and -192) were altered by unloading of the gastrocnemius muscle for seven days, despite a ~4 fold increase in MHC IIb mRNA in the gastrocnemius following this procedure. This could be interpreted as support for the hypothesis that upregulation of the mouse MHC IIb gene in the gastrocnemius is regulated by mRNA stability. Recently published observations have demonstrated that the MHC IIb gene is downregulated dramatically in rat skeletal muscle within hours of initiation of increased contractile activity. This rapid regulation of the MHC IIb gene supports the notion that mRNA stability may be involved rather than, or in addition to transcriptional control.

Nevertheless, because of the low statistical power of our promoter activity experiments, our conclusions are very tentative. We are planning to extend our observations by measuring transcription rate of the endogenous rat MHC IIb gene in control and unloaded skeletal muscle. To accomplish this, we are currently working to set-up and validate the difficult nuclear run-on procedure for skeletal muscle.

This research has provided an important beginning point towards understanding the molecular regulation of the MHC IIb gene in response to unweighting of skeletal muscle. Future work will focus on the regulation of MHC IIb mRNA stability in response to altered loading of skeletal muscle.
Methods:

Animals: Forty-eight female ICR mice (~30g body mass) divided into two groups:

1) Weight-bearing control (n=24)
2) Hindlimb unloaded for 7 days (n=24)

All experiments were approved by institutional review.

Mouse Hindlimb Unloading: Hindlimb unloading (HU) was performed using a modification of the protocol previously used for tail-suspension of rats (AJP 254: C651, 1988). Mice were raised by the tail such that the hindlimbs were elevated just off the cage floor (this produces ~30° head-down tilt). Control and HU mice had *ad libitum* access to food and water.

After 7 days, HU mice were lowered and immediately anesthetized with an IP injection of a cocktail containing ketamine (73.9 mg/ml), xylazine (3.7 mg/ml), and acepromazine (0.7 mg/ml). Upon removal of the hindlimb muscles, anesthetized mice were killed by cervical dislocation.

Northern Blot Analysis: The abundance of myosin heavy chain (MHC) IIb mRNA was determined in gastrocnemius/planaris complex (Gast/Plan), soleus and extensor digitorum longus (EDL) muscles from control and HU mice. Isolated RNA (10 µg) from each muscle was loaded onto a denaturing 1% agarose gel and electrophoresed at 5V/cm for 2 hrs. The RNA was then transferred to a nylon membrane by capillary action and UV crosslinked to the membrane.

A mouse MHC IIb cDNA probe was prepared by reverse transcriptase PCR (GeneAmp RNA PCR kit, Perkin Elmer) using RNA isolated from mouse skeletal muscle as the template. The oligonucleotide primers, 5'-TGTCCTTTCCCTAACCCCTTTAA3' and 5'-GGCTGCGGGCTATTGTGGCACGCT-3', were derived from the MHC IIb cDNA sequence (R.G. Whalen, personal communication) and yield a 111 bp probe corresponding to the first 3 exons of the gene, which are non-coding. The probe was radiolabeled with 32P dCTP via random priming.

The MHC IIb probe and a probe for mouse 18S RNA were separately hybridized to the membrane and visualized by autoradiography. The autoradiographs were analyzed by densitometric scanning. The MHC IIb signal for each sample was then divided by the signal for 18S in the same lane to correct for errors in loading or transfer.

Somatic Gene Transfer: Standard molecular biology techniques were used to subclone the -1430 to +13 bp, -675 to +13 bp and the -192 to +13 bp fragments of the MHC IIb promoter into the pGL3-basic plasmid vector thereby forming chimeric gene constructs in which the MHC IIb promoter fragment drives expression of the reporter gene, firefly luciferase. These chimeric genes were introduced into the Gast/Plan of mice using a modification of the direct plasmid injection technique described by Carson et al. (AJP 268: C918, 1995). Mice were anesthetized as described before for HU. A single incision was made in the skin overlying the Gast/Plan, and a 30 gauge needle was inserted into the midbelly of the muscle to deliver ~75 µg of MHC IIb promoter-Lu plasmid and ~75 µg of a plasmid containing the CMV viral promoter driving expression of the bacterial CAT gene. Plasmid DNA was suspended in 25 µl of a 5% polyvinyl pyrroldione solution. The CMV promoter is not affected by HU; therefore, its activity was used to correct for variations in plasmid uptake efficiency. Following the HU protocol, muscles were analyzed for luciferase and CAT activity as described previously (AJP 268: C918, 1995).

Statistics: Body mass was analyzed using a 2 x 2 ANOVA with repeated measure. Student's t-tests were performed to compare control vs. HU groups for all other dependent variables. Level of significance was established as P < 0.05.

Results:

Seven days of HU resulted in a significant reduction in body mass (Fig. 1). The mass of the Gast/Plan and the soleus muscles were also reduced in the HU mice. This muscle atrophy remained significant even when the muscle masses were expressed as a percentage of post-HU body mass. Mass of the EDL was not affected by the HU treatment (Fig. 1).

Myosin heavy chain IIb mRNA levels (per µg of total RNA) in the Gast/Plan and soleus were elevated ~4 fold and ~12 fold, respectively (Fig. 2).

The activity of the mouse MHC IIb promoter/luciferase constructs in the Gast/Plan did not differ between control and HU groups (Fig. 3).

Conclusions:

1. Seven days of hindlimb unloading in the mouse results in a loss of body mass (~10%). There is also a significant atrophy of the gastrocnemius/plantaris complex (~16%) and soleus (~23%) muscles compared to weight-bearing controls.

2. Unloading of the hindlimb muscles results in an upregulation in the expression of myosin heavy chain (MHC) IIb mRNA in the Gast/Plan and soleus muscles.

3. In vivo deletion analysis of MHC IIb promoter/reporter gene chimeric constructs suggest that the promoter activity is not increased following 7 days of HU.

4. These data suggest that unloading-induced increases in MHC IIb mRNA may involve a change in mRNA stability rather than a direct action of the unloading stimulus on promoter activity and transcription rate.
Figure 1. Changes in body and muscle mass in weight-bearing control and hindlimb unloaded mice.
Figure 2. Fold change (relative to weight-bearing controls) in myosin heavy chain (MHC) IIb mRNA in selected muscles of mice hindlimb unloaded for 7 days.
Figure 3. Myosin heavy chain IIb promoter activities in gastrocnemius muscles of weight-bearing control and hindlimb unloaded (HU) mice.